Bacillus cereus acid stress responses

Maarten Mols

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Thesis

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Preface

At six years of age our son already showed great determination: he wanted to become a biologist so that he could leave for Canada at his sixteenth to assist Grizzly Adams with his bears. Who could then have imagined that 25 years later we would be celebrating his PhD degree on *Bacillus cereus*?
Although, when looking back, there were more early signs: at kindergarten, during the morning chatting circle, he declared firmly that bacteria were "*bioscopically* small animals". When strolling through the woods he gathered all that he could find: twigs, pebbles, leaves and so on. Back at home he left all these objects of investigation near the front door and forgot he ever laid them there, very much like a genuine distraught professor.
A necessary asset for realising this dream was, however, the ability to read. Teaching him to read could rightfully be called an ambitious project as it took a lot of patience from both his teacher as well as his mom. Not only did he seem to be having difficulties in distinguishing the differences between b, d and p (little drawings had to be made), but in addition he wanted to ensure everything he read had effectively the correct meaning: was the little duck he read about also presented on the picture?
When he finally got the hang of reading, his school career was a piece of cake. Favourite subjects were biology and chemistry. Subjects that were followed with a considerable lesser level of enthusiasm were foreign languages. In his view, languages had nothing to do with intelligence, but were just a simple matter of learning by heart.
To finish his masters he ventured outside Utrecht University for a traineeship. His mother, who works at Wageningen UR, advised him to look at laboratories of various institutes in Wageningen. He landed a traineeship placement at RIKILT where he performed well and immediately after finishing he was offered to start with the PhD project "Microbial food preservation and safety. Identification of molecular targets for preservation stresses in *Bacillus cereus*".
Even during this PhD study his family members have proven to be of assistance once again and it turned out to be a huge undertaking for them too. His beloved Brenda nourished and cuddled him, his dad took him fishing to relieve his mind of the stresses and his mom patiently listened to all his frustrations.
And now we are proudly attending the conclusion of his achievement, convinced our son was helped by the right surroundings and a tiny bit of help from his family and friends.

Mr. and Mrs. Mart and Liesbeth Mols

Contents

Abstract

Bacillus cereus is a ubiquitous Gram-positive organism, which frequently causes food-borne illnesses. The widespread prevalence of *B. cereus* makes it a common contaminant in fresh foods where it also can cause spoilage. To prevent food-borne diseases and food spoilage, foods are often processed and/or preserved. In recent years, consumers' preferences have directed to fresher and tastier foods and this has acted as a driver for food industry to use milder processing and preservation techniques. Examples of hurdles that can be applied to preserve foods are low pH and the addition of organic acids. *B. cereus* may overcome these adverse conditions by displaying an adaptive stress response. The response of *B. cereus* upon exposure to these hurdles was investigated using two model strains, ATCC 14579 and ATCC 10987. Comparative analysis revealed numerous strain-specific genes and differences in metabolic capacities, including a urease encoding gene cluster in ATCC 10987 and a nitrate respiration cluster in ATCC 14579. A survey including ATCC 10987 and 48 environmental and outbreak-associated isolates revealed urease activity, i.e., the conversion of urea in ammonia and carbon dioxide, to be present in 10 isolates. However, the activity appeared to be too low to contribute to acid resistance in the strains tested. To search for other acid resistance mechanisms, comparative phenotype and transcriptome analyses of strains ATCC 14579 and ATCC 10987 cells exposed to organic and/or inorganic acid shocks were performed. Upon exposure to low pH with or without the addition of lactic acid or acetic acid, common acid resistance mechanisms and induction of the nitrate reductase cluster in the more acid resistant strain ATCC 14579 were revealed. Furthermore, a major oxidative response was displayed, which included the induction of several oxidative stress related genes and the production of inactivation-associated reactive oxygen species (ROS), such as hydroxyl radicals, peroxynitrite, and superoxide. ROS were detected using fluorescent probes in combination with flow cytometry, including a newly developed method using a specific probe that enables superoxide detection in Gram-positive and Gram-negative bacteria. The formation of ROS was also shown upon exposure to heat and was found to be oxygen dependant. Correspondingly, assessment of *B. cereus* stress survival capacity revealed increased heat- and acid-resistance with cells grown and exposed to stresses in the absence of oxygen. The excess ROS may originate from stress-induced dysfunction of the aerobic electron transfer chain, which was indicated by the induction of alternative electron transfer chain components upon exposure to organic and inorganic acid shocks. Upon exposure to stress, superoxide is generated through the premature leakage of electrons to oxygen at sites in the electron transfer chain at elevated rates. Subsequently, superoxide may promote the formation of other ROS, which can cause cellular damage leading to cell death. The induction of oxidative stress related genes has been reported in numerous other studies involving a wide range of bacteria exposed to different adverse conditions. However, a clear relation between the formation of ROS and the applied environmental stress was up to now not established. Secondary oxidative

responses, including the formation of ROS, are possibly common bacterial responses to severe stresses under aerobic conditions. This thesis describes genomic differences between *B. cereus* strains and the acid stress response of these strains on transcriptome and phenotype levels, including measurements of intracellular ROS. The findings in this study can contribute to further understanding of bacterial stress responses and secondary oxidative responses. Furthermore, the results obtained may aid to optimize and select (combinations of) stresses to apply in hurdle technology, thus enabling design of safe, milder food processing and preservation techniques.

Chapter 1

General introduction and thesis outline

Abstract
Bacillus cereus is a Gram-positive spore-forming bacterium that is ubiquitously found and can cause two types of food-borne illnesses, emesis and diarrhoea and occasionally it causes serious infections. *B. cereus* is also an important spoilage bacterium in for example the dairy industry. To prevent food-borne diseases and spoilage, food is often processed and/or food preservatives are added to maintain product quality and extend the shelf-life. Nowadays, food is often preserved by using a series of stresses, called hurdles, to ensure microbial safety and maintain food quality. Bacteria, such as *B. cereus*, may survive food processing and overcome hurdles by displaying an adaptive stress response. Low pH and/or the addition of organic acids are used to preserve food and these may trigger an acid stress response in *B. cereus*. Analysis of microbial acid stress responses showed that mechanisms of acid resistance can be divided in four main groups, damage repair, metabolic rearrangements, proton transport, and proton consumption. This thesis describes the research on the acid stress response of *B. cereus* strains ATCC 14579 and ATCC 10987. This chapter introduces the work described and includes an outline of the thesis.

Bacillus

The genus *Bacillus*, belonging to the phylum Firmicutes, comprises a diverse group of Gram-positive and Gram-variable bacteria. All members of this genus are rod-shaped organisms that live as single or chained cells of 0.5 to 1.2 µm by 2.5 to 10 µm. *Bacillus* species are aerobic or facultative anaerobic organisms and can form endospores that can be isolated from a wide range of environments (Claus and Berkeley, 1986). *Bacillus subtilis* is used as model organism for Gram-positives and was one of the first organisms of which the complete genome was published (Kunst *et al.*, 1997). Currently, the *Bacillus* genus is one of the most sequenced genera, with 25 published complete genomes and 116 ongoing projects (July 1 2009, GOLD, (Liolios *et al.*, 2008)). The available genome sequences show that the *Bacillus* genus is a diverse group and have a low GC content. The size of the genomes varies from 2790 kb (*Bacillus coagulans* 36D1) to 6685 kb (*Bacillus cereus* 03BB108). The diversity of the strains with corresponding relevancies, ranging from agricultural, food, and biotechnical to medical and even bioterrorism, are the main drivers for sequencing so many strains. Several *Bacillus* species are known for their industrial relevance, because they can excrete large quantities of proteins, such as amylases and proteases. Furthermore, some bacilli are used to ferment food, such as natto (Murooka and Yamshita, 2008), and some are used as probiotic (Sorokulova *et al.*, 2008). Nonetheless, *Bacillus* species are often associated with various types of diseases, indicating the clinical relevance of the *Bacillus* genus. *B. subtilis*, *Bacillus pumilus*, *Bacillus sphaericus*, *Bacillus licheniformis* and other non-*Bacillus cereus* group bacilli have rarely been associated with bacteraemia or septicaemia in predominantly immuno-compromised people (Blue *et al.*, 1995; Castagnola *et al.*, 2001; Ozkocaman *et al.*, 2006; Bentur *et al.*, 2007; Farhat *et al.*, 2008). Several of these *Bacillus* species have also been implicated with food-borne outbreaks and toxin production (Salkinoja-Salonen *et al.*, 1999; From *et al.*, 2005; Pavic *et al.*, 2005; From *et al.*, 2007; Apetroaie-Constantin *et al.*, 2009; Stickel *et al.*, 2009). Notably, the medical relevance of species belonging to the *Bacillus cereus* sensu lato group is more significant, because the number of reported outbreaks and severity of infections is much larger compared to non-*B. cereus* group bacilli.

***Bacillus cereus* group**

The *B. cereus* sensu lato group consists of six different species, *B. cereus*, *Bacillus thuringiensis*, *Bacillus anthracis*, *Bacillus weihenstephanensis*, *Bacillus mycoides*, and *Bacillus pseudomycoides* (Jensen *et al.*, 2003). The cells are relatively large (cell width >1 µm) and most strains are motile. However, several non-motile strains have been described including most *B. anthracis* strains. The species in the *B. cereus* group are grouped based on shared specific genetic elements not present in other *Bacillus* species (Tourasse *et al.*, 2006) and because the strains are genetically closely related (Ash *et al.*, 1991). The phylogenetic relation between different members of the *B. cereus* group is shown in Fig. 1. Due to the close relatedness and based on genetic evidence, it has been proposed that at least *B. cereus*, *B. anthracis* and *B. thuringiensis* could be considered as one single species

(Helgason *et al.*, 2000). Despite their close relatedness, the different species within the *B. cereus* group have very different phenotypic characteristics and medical relevancies, due to harbouring distinct large plasmids encoding for these characteristics (Jensen *et al.*, 2003; Ehling-Schulz *et al.*, 2006; Andrup *et al.*, 2008).

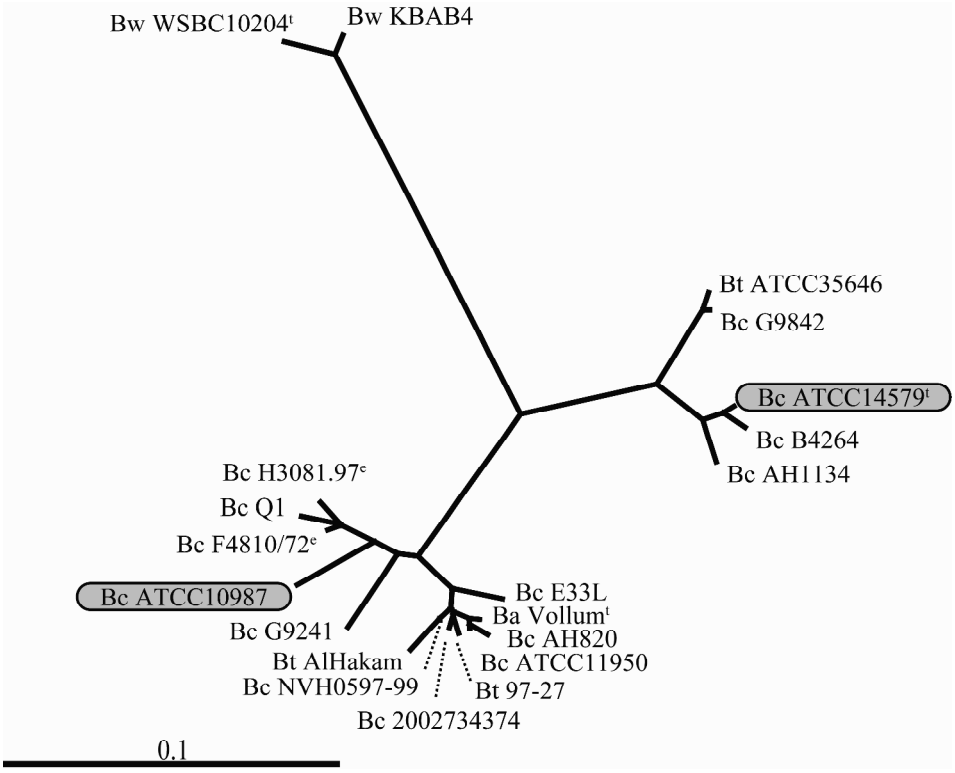

Fig. 1. Neighbour-joining phylogenetic tree of completely sequenced strains and the type strains of the *Bacillus cereus* sensu lato group. The tree shows four main lineages; a cold-tolerant lineage, including *B. weihenstephanensis*, a lineage, including ATCC 14579, mainly consisting of environmental isolates, a *B. anthracis*-like lineage, and an emetic-like lineage. All sequenced *B. anthracis* strains cluster in a single lineage represented by *B. anthracis* Vollum. The tree is based on the sequence of a set of housekeeping genes and the multi locus sequence typing scheme developed by Candelon *et al.* (2004) and Sorokin *et al.* (2006). The tree was constructed using the University of Oslo's *Bacillus cereus* group MultiLocus and MultiData Typing website http://mlstoslo.uio.no (Tourasse and Kolsto, 2008). Ten percent divergence scale is shown at the bottom of the figure. The emetic *B. cereus* strains are indicated with ^{e} and the type strains are indicated with ^{t}. Abbreviations used: Bc: *B. cereus*, Bt: *B. thuringiensis*, Ba: *B. anthracis*, and Bw: *B. weihenstephanensis*.

B. thuringiensis is an insect pathogen (Aronson *et al.*, 1986) and is widely used as insecticide (Aronson and Shai, 2001). Despite this common use, it is known to cause

wound, lung, and eye infections (Samples and Buettner, 1983a, b; Hernandez *et al.*, 1998; Ghelardi *et al.*, 2007) and food-borne disease (Jackson *et al.*, 1995; McIntyre *et al.*, 2008; Ankolekar *et al.*, 2009).
B. anthracis is the causative agent of anthrax, an acute mammalian disease that can present three clinical forms: cutaneous, pulmonary, and gastro-intestinal. The cutaneous form involves skin lesions and is still common in developing countries (van den Enden *et al.*, 2006; Ozcan *et al.*, 2008; Chraibi *et al.*, 2009). The gastro-intestinal form is rare and resembles the cutaneous form by lesions inside the human GI-tract (Beatty *et al.*, 2003; Kanafani *et al.*, 2003; Babamahmoodi *et al.*, 2006). The pulmonary or inhalational form consists of two distinct stages. The first stage of flu-like symptoms is followed by a second stage of systemic infections (Brachman, 1980). This form of anthrax is very rare (Walsh *et al.*, 2007), however bioterrorism attacks and threats have raised the attention towards *B. anthracis* (Jernigan *et al.*, 2001). Of the three forms of disease, pulmonary anthrax has the highest mortality rate (40% to 97% depending on antibiotic treatment), followed by gastrointestinal anthrax (less than 40% with antibiotic treatment), and cutaneous anthrax (1% to 20% depending on antibiotic treatment) (Beatty *et al.*, 2003).
B. cereus is a soil-dwelling organism (Stenfors Arnesen *et al.*, 2008), but especially known as food-borne human pathogen also capable of causing non-gastro-intestinal diseases (Drobniewski, 1993; Kotiranta *et al.*, 2000). It can cause pneumonia in immuno-compromised people (Frankard *et al.*, 2004; Katsuya *et al.*, 2009) and metalworkers and is in rare cases fatal (Hoffmaster *et al.*, 2006; Avashia *et al.*, 2007). Another serious condition *B. cereus* may cause is neonatal meningitis that can also be fatal (Chu *et al.*, 2001; Evreux *et al.*, 2007; Manickam *et al.*, 2008). Furthermore, fatal cases of *B. cereus*-induced bacteraemia (Carretto *et al.*, 2000; Orrett, 2000), septicaemia (Musa *et al.*, 1999; Ginsburg *et al.*, 2003), endocarditis (Cone *et al.*, 2005), and peritonitis (Latsios *et al.*, 2003) have been described in recent years. More common, but mostly much less severe, are food-borne infections and intoxications caused by *B. cereus* and those are discussed in the next sections.

Bacillus cereus
B. cereus was first described in 1887 by Frankland and Frankland (1887). They described smooth, wax-like colonies on agar plates hence the name cereus which is derived from cera, the Latin word for wax. *B. cereus* is a facultative anaerobic organism with large vegetative cells typically 1.0 µm by 3 µm to 5 µm. Mesophilic strains of *B. cereus* can grow in a temperature range between 8 °C and 55°C, with an optimum growth rate between 28 °C and 35 °C. Several psychrotolerant strains have been described that can grow at temperatures as low as 5 °C. Although *B. cereus* may be exposed to adverse conditions in its natural habitats, it does not have a marked tolerance for low pH (pH 5.0 to pH 6.0 depending on acidulant) and water activity (minimal a_w 0.95) (Adams and Moss, 2000). However, the ability to form endospores and the ability to display adaptive stress responses makes *B. cereus* a survival expert. The spores of *B. cereus* are survival vehicles produced

upon nutrient shortage and are metabolically inactive (de Vries *et al.*, 2004). Spores are extremely resistant to stress conditions, such as radiation, high temperature, freezing, drying, and exposure to all kinds of substances, such as cleaning agents (Setlow, 2006). When spores encounter more favourable conditions, they can germinate into vegetative cells and subsequently grow (Hornstra *et al.*, 2006).

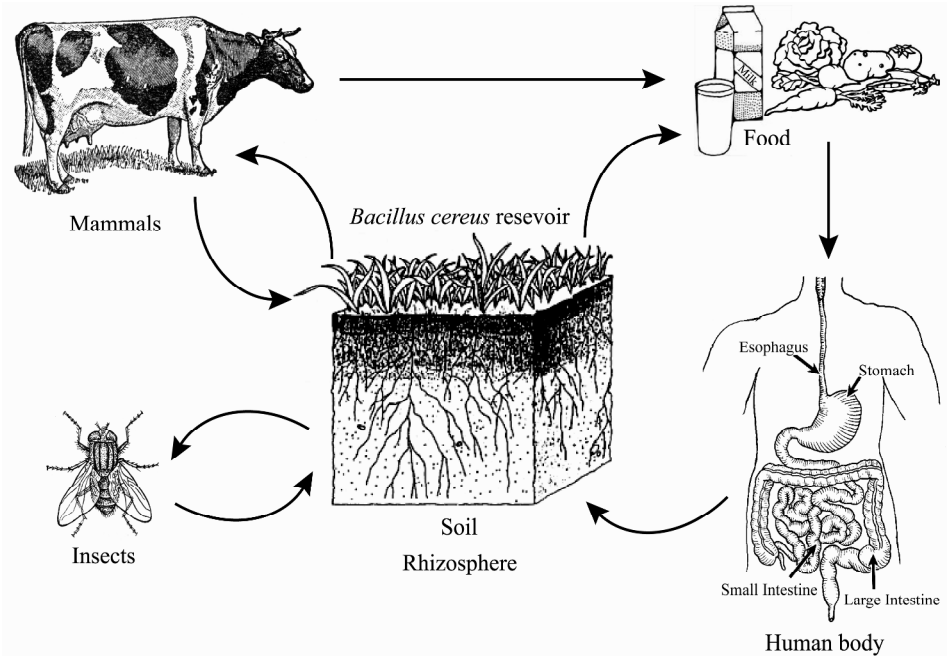

Fig. 2. Illustration of environments from which vegetative cells and/or spores of *B. cereus* can be isolated.

The spores and vegetative cells of *B. cereus* can be found in a wide variety of environments (Fig. 2). The saprophytic lifestyle of *B. cereus* in soil is regarded as one of its main ecological niches (Jensen *et al.*, 2003; Vilain *et al.*, 2006; Stenfors Arnesen *et al.*, 2008). From soil (von Stetten *et al.*, 1999), rhizosphere (Berg *et al.*, 2005) and vegetation (Adams and Moss, 2000) it can easily spread to animals, such as insects (Luxananil *et al.*, 2001) and mammals (Swiecicka *et al.*, 2006; Stenfors Arnesen *et al.*, 2008). Furthermore, *B. cereus* can be transmitted in to raw foods such as milk (Bartoszewicz *et al.*, 2008; Banyko and Vyletelova, 2009), rice (Ankolekar *et al.*, 2009), meat (Smith *et al.*, 2004), soybean sprouts (Kim *et al.*, 2004), and vegetables (Kramer and Gilbert, 1989; Choma *et al.*, 2000). Raw food may be consumed by people, however, often food is processed before consumption. Especially the spores of *B. cereus* are able to survive many food processing steps and therefore, together with recontamination, *B. cereus* can be found in processed foods, such

as pasteurized milk (Lin *et al.*, 1998; Eneroth *et al.*, 2001), cheese (Pirttijarvi *et al.*, 1998), rice dishes (Dufrenne *et al.*, 1994), pasta (Dierick *et al.*, 2005), cooked vegetables (Guinebretiere *et al.*, 2003), cooked-chilled foods (Choma *et al.*, 2000), and ready-to-eat-meals (Rosenquist *et al.*, 2005; Wijnands *et al.*, 2006). Upon ingestion of contaminated food, *B. cereus* can enter the human gastro-intestinal tract (Turnbull and Kramer, 1985) and may cause food-borne illnesses (Kotiranta *et al.*, 2000).

B. cereus induced food-borne diseases

Nowadays *B. cereus* is a known food-borne human pathogen that frequently causes illnesses. The association of *B. cereus* with food-borne diseases was proposed in 1906, when staff and inmates of a sanatorium developed diarrhoea (Lubenau, 1906). In 1950, *B. cereus* was conclusively established as a cause of food-borne illness (Hauge, 1950). Additionally, Hauge artificially contaminated sterile vanilla sauce with a *B. cereus* strain isolated from an outbreak and ate the sauce. After 16 hours, *B. cereus*-attributed diarrheal symptoms were developed (Hauge, 1955). Besides this diarrheal type of food-borne disease, *B. cereus* can cause another type of illness; emesis. The emetic type of food poisoning was firstly attributed to *B. cereus* in 1974 when 13 persons showed emetic symptoms after eating fried rice (Mortimer and McCann, 1974).

The diarrheal type is a toxico-infection caused by enterotoxins produced inside the human small intestine (Granum, 1994). The toxins are produced on site by vegetative cells that were either ingested as vegetative cells or as spores that after ingestion germinate in the gastro-intestinal tract (Wijnands *et al.*, 2007). The typical symptoms are abdominal pain, watery diarrhoea and occasionally nausea and emesis. Therefore, the *B. cereus*-induced toxico-infection is easily confused with the food-borne disease caused by *Clostridium perfringens* (Granum, 1990). Normally the symptoms are relatively mild and self-limiting, however, a few lethal cases have been reported (Lund *et al.*, 2000; Schoeni and Wong, 2005). The incubation time generally ranges from 8 to 16 hours. Normally the symptoms last for 12-24 hours, but a duration of several days has been reported in some cases (Kramer and Gilbert, 1989). Generally, relatively high numbers of cells, i.e., 10^5-10^8 viable counts per gram food, are associated with the diarrheal symptoms. However, in some cases viable counts as low as 200 cfu g^{-1} food have been associated with disease (Stenfors Arnesen *et al.*, 2008). Furthermore, the presence of spores in food may lower the infectious dose, because spores easily survive the stomach transit and may geminate and grow inside the human intestine (Clavel *et al.*, 2004). Especially foods with high protein contents, including dairy products, meat, chicken, vegetables, and seafood, are implicated with *B. cereus* toxico-infections and several enterotoxins and putative virulence factors have been described (Schoeni and Wong, 2005; Stenfors Arnesen *et al.*, 2008). The most well-known enterotoxins are haemolysin BL, non-haemolytic enterotoxin and cytotoxin K. Notably, these enterotoxins do not always co-exist in *B. cereus* strains. Haemolysin BL is encoded on the chromosome by *hblCDAB* and consists of three different proteins, a B component and two L components. Individually, the three components of HBL are non-toxic, but

together they form a complex, which causes lysis of cells by forming trans-membrane pores (Beecher and Wong, 1997). Non-haemolytic enterotoxin is also a tri-partite, pore forming protein complex. It consists of subunits NheA, NheB and NheC and the corresponding genes are also located on the chromosome (Fagerlund *et al.*, 2008). Cytotoxin K is haemolytic and toxic towards human intestinal Caco-2 cells (Guinebretiere *et al.*, 2002; Fagerlund *et al.*, 2004). It was first isolated from a *B. cereus* strain which was the causative agent of a lethal gastro-enteritis outbreak in France (Lund *et al.*, 2000). Cytotoxin K is encoded by *cytK* on the chromosome and can form pores in membranes (Hardy *et al.*, 2001).

Table 1. Characteristics of the two types of food-borne illnesses caused by *B. cereus (adapted from Stenfors Arnesen et al., 2008)*

Characteristic	Diarrheal type	Emetic type
Type of toxin	Protein, protein complex	Dodecadepsipeptide
Toxin genes	e.g. *hblCDAB*, *nheABC*, *cytK*	*cesHPTABCD*
Location of toxin genes	Chromosome	Plasmid
Toxin formation	Ribosomal	Non-ribosomal
Action of toxin	Pore formation	Potassium ionophore
Location of toxin production	Host small intestine	Food
Toxin stability	Heat and pH unstable	Heat and pH stable
Infective dose	10^5-10^8 cfu g^{-1}	10^5-10^8 cfu g^{-1} found in outbreak associated foods
Symptoms	Watery diarrhoea, abdominal pain, occasionally nausea	Vomiting, nausea, malaise
Incubation time	8-16 h	0.5-6 h
Duration of symptoms	12-24 h	6-24 h
Associated foods	Proteinaceous foods, such as meat products, soups, vegetables, puddings, sauces, and milk	Starch rich foods, such as fried and cooked rice, pasta, and pastry products

The emetic type of illness is caused by the emetic toxin, cereulide (Table 1). Cereulide is a 1.2 kDa cyclic peptide, structurally related to valinomycin, containing two amino acids and two oxyacids (D-*O*-Leu-D-Ala-L-*O*-Val-L-Val)$_3$ (Adams and Moss, 2000). It is pre-formed in food and because it remains stable upon heat and acid exposures, the toxin is still active after cooking and stomach transit (Kramer and Gilbert, 1989). Upon ingestion of cereulide typical symptoms, resembling *Staphylococcus aureus* intoxication (Le Loir *et al.*, 2003), such as nausea, vomiting and general malaise may arise in 0.5 to 6 hours. The symptoms are mild, however in rare cases subsequent liver failure can be lethal (Mahler *et al.*, 1997; Dierick *et al.*, 2005). Generally the symptoms last for 6 to 24 hours (Ehling-Schulz *et al.*, 2004). The number of *B. cereus* cells required to produce intoxicating levels of cereulide has not been determined. However, foods that have been associated with outbreaks of the emetic type of disease contained in most cases between 10^5 and 10^8 cfu g^{-1} (Kramer and Gilbert, 1989). Using animals and Hep-2 cells, a minimal emesis-causing dose of cereulide of 8 to 10 µg kg^{-1} body weight was established (Agata *et al.*, 1994; Shinagawa *et al.*, 1995).

Mainly foods such as pasta and rice dishes are associated with outbreaks of *B. cereus*-induced emetic syndromes (Schoeni and Wong, 2005). However, recently emetic strains have been isolated from milk and infant formula (Shaheen *et al.*, 2006), but no dairy-associated outbreaks of emesis have been reported. The emetic toxin of *B. cereus* is synthesized by cereulide synthetase, which is encoded by the genes *cesHPTABCD* located on a large plasmid (Ehling-Schulz *et al.*, 2006). The presence of this large plasmid and hence cereulide production are restricted to a single evolutionary lineage within the *B. cereus* group (Ehling-Schulz *et al.*, 2005b). In contrast, enterotoxin genes are more common within the *B. cereus* group and are also often present in emetic *B. cereus* strains and *B. thuringiensis* (Kyei-Poku *et al.*, 2007). Another striking difference between enterotoxins and cereulide is the translation of the peptides. Enterotoxins are translated at ribosomes and cereulide is formed by cereulide synthetase and not by ribosomes (Ehling-Schulz *et al.*, 2005a).

Table 2. Number of reported *B. cereus* infections in The Netherlands in 2004-2007

	2007	2006	2005	2004
B. cereus caused incidents[1]	23	22	19	17
Total incidents with known cause	57	56	148	95
Total incidents	564	474	387	506
Percentage *B. cereus* incidents[2]	41%	39%	13%	18%

[1] An incident is a food-borne outbreak with more people or a single case

[2] Percentage given is number of *B. cereus* incidents in relation to the total incidents with a known cause

The prevalence of *B. cereus* induced food-borne illnesses is difficult to determine, because the symptoms associated with *B. cereus* infections or intoxication are generally mild. Therefore, it is conceivable that many *B. cereus* infections are not reported and that the prevalence of these infections is largely underestimated. Generally, *B. cereus* cases and outbreaks are only reported when either a large number of patients acquired symptoms or when the symptoms are more severe than usual. Despite the possible underestimation, *B. cereus* was the cause of most incidents with an identified agent in The Netherlands in 2007 (Doorduyn *et al.*, 2008). Between 2004 and 2007, *B. cereus* was the causative agent of food-borne illnesses in percentages ranging from 13% to 41% (Table 2). A comprehensive surveillance study in Europe has shown that in some countries, most notably Norway and The Netherlands, *B. cereus* is an important causative agent of food-borne infections (Schmidt and Gervelmeyer, 2003). In Norway similar data were reported as for The Netherlands, 35% and 32% of outbreaks were caused by *B. cereus* in 1999 and 2000, respectively. In other countries, lower relative incidences of outbreaks of *B. cereus* were reported. England, Italy, Germany, and France reported numbers ranging from 0.5% to 5% for *B. cereus*-attributed outbreaks in 1999 and 2000, respectively. The differences in reported incidences between countries may be caused by different food consumption patterns or different food handling. However, differences in local procedures for *B. cereus*

detection and awareness of *B. cereus* infections are more likely causing the large differences in reported *B. cereus*-attributed cases between countries.

B. cereus food spoilage

Besides being an important food-borne pathogen, *B. cereus* is also a notorious food spoilage organism. Food spoilage is caused by growth of unwanted bacteria in food and causes enormous expenses for food industry (Gram *et al.*, 2002). *B. cereus* causes mainly spoilage of milk and dairy products, thereby shortening the shelf-life of these products. Raw milk is often contaminated with *B. cereus* spores (te Giffel and Beumer, 1998) and these spores can easily survive pasteurization and germinate in milk (Wilkinson and Davies, 1973). Furthermore, spores and vegetative cells of *B. cereus* can attach to processing equipment and food contacting surfaces and subsequently form biofilms. Biofilms are multi-cellular complexes within a matrix of polysaccharides that are attached to a surface. Cells that are embedded in a biofilm tend to be more resistant to cleaning agents, making them hard to eradicate from processing equipment (Peng *et al.*, 2002; Stoodley *et al.*, 2002). Furthermore, biofilms may lead to equipment failure and corrosion (Beech *et al.*, 2005). From biofilms, cells and spores are easily dispersed and therefore biofilms are a continuous source of (re)contamination (Wijman *et al.*, 2007). Especially when dispersed cells and spores can germinate and grow spoilage can occur. Several *B. cereus* strains are known to grow at low temperatures and growth and subsequent spoilage may occur rapidly when the refrigerator temperature is abused. Therefore, the economic impact of unwanted *B. cereus* growth in food and food processing lines is substantial (te Giffel, 2001).

Stress response

To prevent spoilage and food safety issues, food is often processed and preserved. The application of certain preservation techniques result in the inactivation or prevention of growth of microorganisms in food. Classically food was sterilized by heat, however, nowadays consumers' demands made the food industry to use milder conditions for preservation (Abee and Wouters, 1999). Intelligent selections, combinations, and applications of different preservation steps, called hurdles, is frequently applied in modern food industries to ensure the microbial safety and stability as well as the sensory and nutritional quality (Leistner, 2000). Mild heat treatments, a low pH, and high osmotic values are examples of hurdles used to preserve food. The response of bacteria to these adverse conditions may lead to the possibility that bacteria overcome the applied stress conditions and hurdles. Therefore, the stress response of *B. cereus* has been studied in detail.

The heat and salt stress response and stress adaptation of *B. cereus* was investigated by Browne and Dowds (2001). They found that chaperone GroEL was induced upon heat stress exposure and a role for glycolysis in adaptation to heat shock and osmotic stress was revealed. The heat stress response was investigated in more detail, describing numerous

proteins, such as DnaK, SodA, ClpP, and RsbV, to be involved (Periago *et al.*, 2002). Furthermore, the role of alternative sigma factor σ^b in the stress response and heat adaptation was investigated, showing that σ^b is an important factor for *B. cereus* to adapt to adverse conditions (van Schaik *et al.*, 2004) and may have an impact in food preservation and safety (van Schaik and Abee, 2005). Besides the heat stress response also the response of *B. cereus* to salt stress has been studied in detail. *B. cereus* shows to adapt to high salt concentrations making it more resistant to subsequent stresses (Browne and Dowds, 2001; den Besten *et al.*, 2006). General stress response involved genes, such as *sigB* and *clpC*, and osmolytes transporters were found to be involved in the response of *B. cereus* to salt stress (den Besten *et al.*, 2009).

In contrast to the salt and heat stress response of *B. cereus*, the acid stress response has not been studied extensively. Available information is limited to alternative sigma factor σ^B expression upon exposure to low pH (van Schaik *et al.*, 2004) and the acid tolerance response, which includes modulation of intracellular pH and protein synthesis (Browne and Dowds, 2002; Jobin *et al.*, 2002; Thomassin *et al.*, 2006). Notably, up to now the underlying molecular mechanisms of the acid stress response of *B. cereus* have not been elucidated.

Acid stress response

Although information on the molecular mechanisms of the acid stress response of *B. cereus* is limited, data on the response of other Gram-positive organisms upon exposure to low pH is available and has been reviewed by Cotter and Hill (2003). Generally, mechanisms of acid resistance can be divided into four groups, i.e., damage repair, metabolic rearrangements, proton transport, and proton consumption (Fig. 3).

Respiring bacteria use their electron transport chain to create a proton gradient in aerobic conditions. The proton motive force (PMF), comprising the membrane potential and the proton gradient, can subsequently be used to generate ATP via F_1F_0-ATPsynthase. Proton translocation by active electron transport chains may also aid in pH homeostasis in lactic acid bacteria, as shown by the reconstituted proton extrusion in an H^+-dependent ATPase negative mutant of *Lactococcus lactis* when haemin was supplied (Blank *et al.*, 2001). F_1F_0-ATPsynthase can, besides generating ATP from the passive inflow of protons, extrude protons at the expense of ATP in several bacteria such as *Streptococcus mutans* (Bender *et al.*, 1986) and *Listeria monocytogenes* (Datta and Benjamin, 1997). Initially, the importance of proton extrusion by F_1F_0-ATPase was established in *Enterococcus hirae*. This organism, formerly known as *Streptococcus faecalis*, does not have a respiratory chain and thus F_1F_0-ATPase is only used for proton extrusion and not for ATP synthesis (Harold *et al.*, 1970). The PMF is used by many transporters to translocate ions, nutrients and other metabolites across the membrane. In neutral conditions, the influx of H^+ is used to import or export other substances. However, proton antiporters may be used to export protons outside the cell in acidic environments (Macpherson *et al.*, 2005; Bore *et al.*, 2007; Wilks *et al.*, 2009). On the other hand, H^+ dependant transporters generally are down-regulated upon

exposure to low pH environments, to prevent the influx of protons lowering the internal pH (Bore *et al.*, 2007; Wilks *et al.*, 2009).

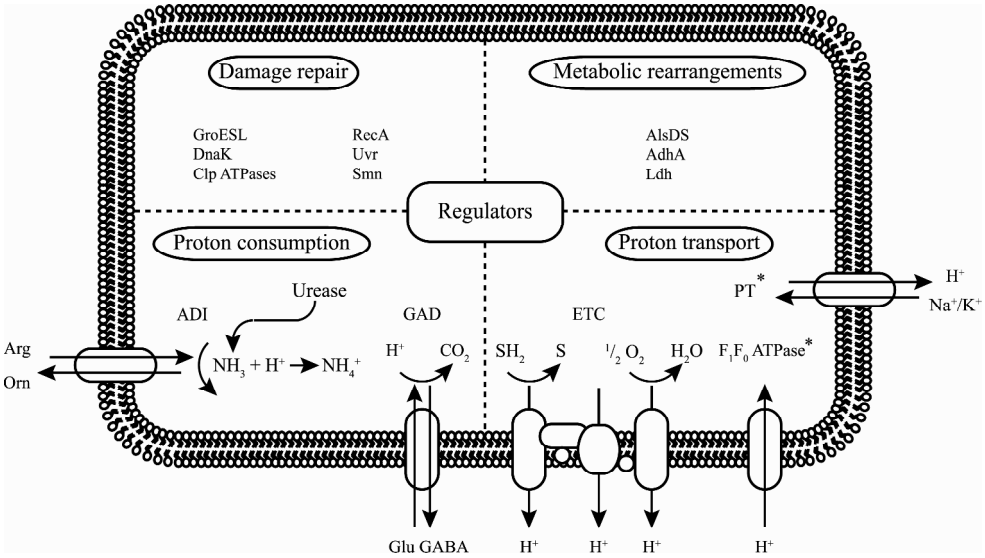

Fig. 3. Graphical representation of acid stress resistance mechanisms in Gram-positive organisms. These have been divided in four different groups: (i) damage repair, (ii) metabolic rearrangements, (iii) proton transport, and (iv) proton consumption. The four different groups may have specific or shared transcriptional regulators. Proton transport involves proton-mediated symporters and antiporters (PT), F_1F_0-ATPase, and the electron transport chain (ETC). Proton dependant transporters and F_1F_0-ATPase may transport protons in both directions, inwards and outwards and are indicated with an asterisk. Three mechanisms of proton consumption are depicted, the arginine deiminase (ADI) pathway and urease, both producing ammonium via ammonia and glutamate carboxylase (GAD) that forms carbon dioxide. Damage repair can be divided into two subgroups, protein and DNA repair. Examples of enzymes involved in protein repair are chaperones GroESL and DnaK and Clp proteases. DNA repair is exemplified by recombinase A (RecA), UV excinuclease (Uvr) and *Streptococcus mutans* exonuclease (Smn). Acetoin production (AlsDS), alcohol dehydrogenases (AdhA), and lactate dehydrogenases (Ldh) are metabolic pathways described to be involved in acid stress responses. Other abbreviations used: S: substrate, Arg: arginine, Orn: ornithine, Glu: glutamate, and GABA: gamma-aminobutyric acid.

Arginine deiminase or the ADI pathway has been identified in a variety of Gram-positive bacteria, including *Bacillus* spp., *L. monocytogenes*, and several lactic acid bacteria (Cunin *et al.*, 1986; Ryan *et al.*, 2009). The ADI pathway converts arginine to citrulline and ammonia. Subsequently, citrulline is metabolised into ornithine generating carbon dioxide, ammonia and ATP. Arginine and ornithine are exchanged via an antiporter importing arginine and exporting ornithine. The generated ammonia is rapidly protonated inside the

cell, consuming protons and forming ammonium. The role of the ADI pathway in acid stress resistance has been established in bacteria such as *L. monocytogenes* and streptococci. *Streptococcus sanguis* showed arginine deiminase activity upon exposure to pH 3.5 leading to increased resistance (Curran *et al.*, 1995) and ADI negative mutants of *L. monocytogenes* were shown to be more sensitive to low pHs (Ryan *et al.*, 2009). Another ammonia producing mechanism associated with low pH resistance is urease. The urease enzyme catalyzes the hydrolysis of urea, generating two molecules of ammonia and one molecule of carbon dioxide. The genes encoding urease are present in the genomes of various organisms, including plants, fungi, and bacteria (Collins and D'Orazio, 1993). The role of urease is well studied in the human pathogen *Helicobacter pylori*. *H. pylori* can colonize the human stomach where it may cause ulcerations. It is capable of surviving these acidic conditions because it expresses large quantities of urease (Tsuda *et al.*, 1994). Also urease activity in *Streptococcus salivarius* contributes to low pH resistance (Chen *et al.*, 2000). Amino acid decarboxylases play a role in pH homeostasis, by converting amino acids in reactions that consumes an intracellular proton. Amino acid decarboxylases generally function in concert with substrate/product antiporters (Bearson *et al.*, 1997). Examples of such mechanisms are the lysine, arginine, and glutamate decarboxylase (GAD) systems that exchange the resultant products cadaverine, agmatine, or gamma-aminobutyric acid, respectively. Of the three systems mentioned, only GAD has been associated with pH homeostasis in Gram-positive organisms and its role has been established in lactic acid bacteria (Sanders *et al.*, 1998; Fernandez and Zuniga, 2006) and in *L. monocytogenes* (Cotter *et al.*, 2001a; Cotter *et al.*, 2001b; Cotter *et al.*, 2005; Francis *et al.*, 2007).

Several metabolic pathways have been associated with growth at low pHs. Pathways, also involved in fermentative growth (Zigha *et al.*, 2006; van der Voort and Abee, 2009), such as lactate (*ldh*), alcohol (*adh*) and butanediol (*alsSD*) production are up-regulated upon low pH growth of *B. subtilis* (Wilks *et al.*, 2009). It is known that AlsSD shunts fermentation into neutral products, minimizing acid production (Ali *et al.*, 2001). Adh is an NAD(P)-dependant dehydrogenase that may remove acidity and transfer electrons to the electron transfer chain (Reid and Fewson, 1994). Ldh may convert lactate to pyruvate to remove acidic compounds, restoring NAD^+/NADH balance and form pyruvate, a main substrate for gluconeogenesis and the TCA cycle (Lin and Iuchi, 1991).

A low pH also induces several proteins that are considered to be part of a general stress response and are associated with damage repair. Exposure to low pHs can result in misfolding of proteins and damage to macromolecules such as DNA. Consequently, bacteria activate repair mechanisms or dispose the damaged intracellular macromolecules. Misfolded proteins are refolded by chaperones GroEL and DnaK or cleaved by Clp proteases. A link between acid stress and chaperones has been demonstrated in a number of other Gram-positive bacteria. Upon low pH exposure GroEL and DnaK are up-regulated in several lactic acid bacteria (Hartke *et al.*, 1996; Lim *et al.*, 2000), *Clostridium perfringens* (Villarreal *et al.*, 2000), and *L. monocytogenes* (Ryan *et al.*, 2008). DnaK was even necessary for optimal acid resistance in *S. mutans* (Lemos et al., 2007) and *Brucella suis*

(Kohler *et al.*, 2002). Clp proteases were up-regulated upon exposure to acid in *S. mutans* (Len *et al.*, 2004) and in *B. suis* it was found that a *clpB* mutant was more sensitive to acid (Ekaza *et al.*, 2001). Damaged DNA can be repaired by nucleotide excision carried out by Uvr proteins. In *S. mutans* (Hanna *et al.*, 2001) and *Lactobacillus helveticus* (Cappa *et al.*, 2005), it has been demonstrated that nucleotide excision by Uvr is part of the response to low pH adaptation. Another DNA repair mechanism has also been associated with acid resistance in *S. mutans*, Smn (*S. mutans* exonuclease) (Hahn *et al.*, 1999). Smn initiates the repair of damage that originates from protonation of DNA bases.

These different mechanisms of acid resistance can be regulated by specific or shared regulators. One of the known regulators that is involved in acid stress adaptation of *S. aureus* and *L. monocytogenes* is σ^B (Wemekamp-Kamphuis *et al.*, 2004; Cebrian *et al.*, 2009) Induction of *sigB* expression upon exposure to low pH has also been established for *B. cereus* (van Schaik *et al.*, 2004). However, a *sigB* deletion mutant of *B. cereus* was not found to be more sensitive to low pHs.

Notably, the acid resistance mechanisms described above have been investigated mainly in lactic acid bacteria, such as lactobacilli and streptococci. Generally, lactic acid bacteria do not display aerobic respiration and create their own acidic environments by fermenting sugars to lactic acid (Brooijmans, 2008). *B. cereus*, on the other hand, respires in aerobic conditions and does not acidify its growth medium by fermenting sugars. Therefore, the acid stress response of *B. cereus* and the accompanying resistance mechanisms may be different from the ones described above.

B. cereus strains ATCC 14579 and ATCC 10987

B. cereus strains ATCC 14579 and ATCC 10987 were used throughout this thesis. *B. cereus* ATCC 14579 (Fig. 4A) is the *B. cereus* type strain and used in laboratories all over the world. It was isolated from air in a cow-shed in 1887 (Frankland and Frankland, 1887). The full genome sequence of ATCC 14579 was the third publically available genome sequence of the *Bacillus* genus and the first from the *B. cereus* group. Its genome harbours different enterotoxin genes, e.g., *nhe*, *hbl* and *cytK*, and this strain does not harbour the plasmid encoding for emetic toxin production. It is known that ATCC 14579 can produce haemolysin BL and non-haemolytic enterotoxin (Fletcher and Logan, 1999). Furthermore, ATCC 14579 is part of a group of mostly environmental *B. cereus* sensu lato strains that in some cases are involved in diarrheal outbreaks (Fig. 1) (Ehling-Schulz *et al.*, 2005b; Tourasse *et al.*, 2006).

B. cereus ATCC 10987 (Fig. 4B), was isolated from a spoiled cheddar cheese in Canada (Herron, 1930) and is up to now mainly used as a reference strain for biofilm formation and surface behaviour research (Auger *et al.*, 2006; Wijman *et al.*, 2007). The full genome sequence of ATCC 10987 and its large plasmid pBC10987 became available in 2004 (Rasko *et al.*, 2004). Interestingly, ATCC 10987 does not harbour the genes encoding for haemolysin BL, while *nhe* and *cytK* are present (Mols *et al.*, 2007). It belongs to a group of *B. cereus* strains often associated with emetic toxin formation (Fig. 1), however, ATCC

10987 does not harbour the emetic toxin genes (Ehling-Schulz *et al.*, 2005b). A striking difference between ATCC 14579 and ATCC 10987 is the cell size, with ATCC 10987 having significantly smaller vegetative cells (Fig. 4).

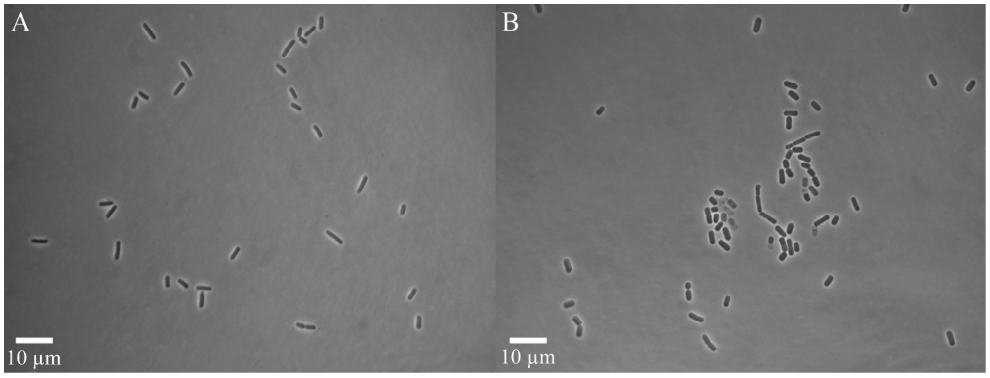

Fig. 4. Phase contrast photograph of vegetative cells of *B. cereus* ATCC 14579 (A) and ATCC 10987 (B). The scale is indicated at the bottom of the figures.

The strains used in this thesis are representatives of two distinct lineages in the *B. cereus* group of which the genomes are fully sequenced. The original papers describing the genomes (Ivanova *et al.*, 2003; Rasko *et al.*, 2004) already described some strain-specific characteristics. These strains were compared throughout this thesis, with the aim to get a more comprehensive understanding of the total array of mechanisms *B. cereus* may display upon acid stress exposure and which mechanisms play a role in acid stress survival.

Thesis outline

The research described in this thesis was initiated to gain a comprehensive insight in the molecular mechanisms behind the acid stress response of *B. cereus*. A better understanding of the behaviour of *B. cereus* may lead to more efficient approaches regarding food preservation. The results described in this thesis may aid in designing safer, mildly preserved foods.

This **Chapter 1** provides an introduction to the thesis and includes an overview of general features of *B. cereus*, its pathogenesis and acid stress responses. Throughout this thesis two sequenced strains from different evolutionary lineages were studied to have a more comprehensive insight in the capabilities of *B. cereus*. First, the genomes and metabolic capacities of strain ATCC 14579 and strain ATCC 10987 were compared in **Chapter 2**, showing numerous differences in nutrient utilization and in genome content between the two strains. One of the most notable differences between the strains was the presence of a urease cluster. Urease is an important enzyme in acid stress resistance of several bacteria. Therefore the role of urease in acid stress response and nitrogen metabolism of *B. cereus*

ATCC 10987 was investigated in **Chapter 3**. The response of ATCC 14579 to pH 5.5 using different acidulants, i.e., hydrochloric acid, lactic acid, and acetic acid was investigated in **Chapter 4**. This study showed that transcriptome profiles of cells exposed to lethal conditions differed from that of cells exposed to mild conditions. Therefore, in **Chapter 5**, a comparative transcriptomic and phenotypic analysis of both strain ATCC 14579 and strain ATCC 10987 exposed to non-lethal and lethal levels of acidity set with hydrochloric acid was performed. A major oxidative response was revealed and lethal pHs could be associated with the formation of reactive oxygen species. In **Chapter 6**, the formation of reactive oxygen species in both strains was tested upon exposure to heat, acid, salt, and hydrogen peroxide stress when the cells were grown and exposed in aerobic, microaerobic, and anaerobic conditions. The generation of these reactive oxygen species may depend on superoxide formation. Therefore, a fluorescent probe for superoxide detection was used to investigate the formation of superoxide in strain ATCC 14579 upon exposure to tellurite, heat and acid in **Chapter 7**. Finally, **Chapter 8** presents a general discussion, concluding remarks and perspectives of the research described in this thesis.

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Chapter 2

Metabolic capacity of *Bacillus cereus* strains ATCC 14579 and ATCC 10987 interlinked with comparative genomics

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Abstract
Bacillus cereus is an important food-borne pathogen and spoilage organism. In this study, numerous phenotypes and the genomes of *B. cereus* strains ATCC 14579 and ATCC 10987 were analysed to compare their metabolic capacity and stress resistance potential. The growth performance of the two strains was assessed for nearly 2000 phenotypes, including use of nutrient sources, performance in acid and basic environments, osmo-tolerance and antibiotic resistance. Several food-relevant phenotypic differences were found between ATCC 14579 and ATCC 10987, such as differences in utilization of carbohydrates, peptides, amino acids and ammonia. Subsequently, the genomes of both strains were analysed with INPARANOID to search for strain-specific open reading frames (ORFs). *B. cereus* ATCC 14579 and ATCC 10987 were found to harbour 983 and 1360 strain-specific ORFs respectively. The strain-specific phenotypic features were interlinked with corresponding genetic features and for several phenotypic differences a related strain-specific genetic feature could be identified. In conclusion, the combination of phenotypic data with strain-specific genomic differences has led to detailed insight into the performance of the two *B. cereus* strains, and may supply indicators for the performance of these bacteria in different environments and ecological niches.

Introduction

Bacillus cereus is a ubiquitously present, spore-forming, motile, Gram positive food-borne human pathogen (Kotiranta *et al*., 2000). Its spores and vegetative cells can be found in environments such as soil (Vilain *et al*., 2006), rhizosphere (Berg *et al*., 2005), foods (Kotiranta *et al*., 2000) and in the air (Frankland and Frankland, 1887). *B. cereus* is specifically associated with foods such as rice, pasta and milk (Dufrenne *et al*., 1994; Rusul and Yaacob, 1995; Larsen and Jorgensen, 1999; Agata *et al*., 2002) and can cause two types of food-borne diseases; emesis and gastro-enteritis (Kotiranta *et al*., 2000). In most cases the symptoms are mild; however, the subsequent systemic infection can be fatal (Dierick *et al*., 2005). *Bacillus cereus* can also cause non-gastrointestinal infections (Drobniewski, 1993), such as periodontitis (Gaur and Shenep, 2001), fulminant endophthalmitis (Beecher *et al*., 2000) and meningitis in immuno-compromised children (Gaur *et al*., 2001). Furthermore, *Bacillus anthracis*, a close relative of *B. cereus*, is the causative agent of anthrax, an acute fatal animal and human disease (Keim and Smith, 2002) and it has been employed as a bioterror agent (Jernigan *et al*., 2002). Both, *B. cereus* and *B. anthracis* are members of the *Bacillus cereus sensu lato* group (*B. cereus*, *B. anthracis*, *Bacillus thuringiensis*, *Bacillus weihenstephanensis*, *Bacillus mycoides* and *Bacillus pseudomycoides*) (Jensen *et al*., 2003).

Recently, four *B. cereus* genome sequences have been published, ATCC 14579 (Ivanova *et al*., 2003), ATCC 10987 (Rasko *et al*., 2004), G9241 (Hoffmaster *et al*., 2004) and E33L (Han *et al*., 2006) and several genomic contigs of other strains are available through GenBank (http://www.ncbi.nlm.nih.gov/). The sequence availability has led to a focus on comparative genomics of the *B. cereus* group and the investigation of the genetic relationship between its members (Zhang and Zhang, 2003; Anderson *et al*., 2005; Rasko *et al*., 2005). These studies have revealed several differences between members of the *B. cereus* group, showing that the main virulence factors of the different members are located on large plasmids.

A number of genomic differences between the enterotoxic *B. cereus* strains ATCC 14579 and ATCC 10987 have been described (Rasko *et al*., 2004; Han *et al*., 2006). Nitrate and nitrite reductase clusters were only found in the genome of ATCC 14579, suggesting that this strain is capable of nitrate respiration under anaerobic conditions (Nakano and Zuber, 1998). Furthermore, gene clusters involved in the metabolism of tagatose and xylose were identified as ATCC 10987 specific. Tagatose, an isomer of galactose, is found in dairy products (Wilhelm and Hollenberg, 1984; Taylor *et al*., 2005) and xylose is abundantly available as a derivative of plant biomass. Finally, *B. cereus* ATCC 10987 contains genes putatively encoding urease, and it has been suggested that the corresponding urea degrading activity could increase the fitness of *B. cereus* ATCC 10987 in acidic environments (Rasko *et al*., 2004). This putative function is based on observations that revealed urease activity to be required for colonization of the human stomach by *Helicobacter pylori* (Tsuda *et al*., 1994).

Jensen and colleagues (2003) have argued that there is a lack of knowledge on the ecology of *B. cereus,* including its symbiotic lifestyle with insects. Moreover, it has been suggested that bacteria -invertebrate interactions may have shaped the evolution of human pathogens (Waterfield *et al*., 2004). The capability of bacteria to utilize available nutrients and the ability to cope with different environments are some of the determining factors in the success of the bacteria to exist in a variety of environments, such as insects' guts. Notably, *B. cereus* is known to grow on a wide range of substrates (te Giffel *et al*., 1997). A broad phenotypic screening such as described for *Staphylococcus aureus* (von Eiff *et al*., 2006) has not been reported, although the need for such information has been expressed recently by Jensen and colleagues (2003). Therefore, this study aims to link comparative genomics data with strain-specific phenotypic features of *B. cereus* strains ATCC 14579 and ATCC 10987. This combination not only displays the strain-specific capabilities, but also allows for the assessment of the impact of specific gene clusters upon strain performance. *B. cereus* strains ATCC 14579 and ATCC 10987 were profiled for almost 2000 phenotypes, such as nutrient usage, growth in high osmolarity, growth at high and low pH and antibiotic resistance. Several differences between these strains, relevant for the performance of *B. cereus* in different environments, were revealed. Combining the phenotypic data with strain-specific genomic differences has led to detailed insight into the performance of the two *B. cereus* strains.

Materials and methods

Bacterial strains
The *B. cereus* strains ATCC 14579 and ATCC 10987 used in this study were obtained from the American Type Culture Collection (ATCC).

Phenotype Microarray
The phenotypic comparison of ATCC 14579 and ATCC 10987 was performed by Biolog Inc. Hayward, CA, USA (http://www.biolog.com). Strain performance was tested in 1920 conditions as described by Bochner and colleagues (2001). In this study, 20 PM 96 well plates were used. Plates PM1 to PM8 were used to test the utilization of metabolic sources, PM9 to investigate the growth at high osmolytes, PM10 to test growth in low and high pH with and without possible protectants and PM31 to PM40 for chemical sensitivity.

Verification tests
Several phenotypes, assessed with the Biolog method, were verified in a minimal medium containing only one carbon and one nitrogen source. All chemicals used in this study were obtained from Merck (Germany) or Sigma (MO, USA). This minimal medium was derived from GGGS medium (Buono *et al*., 1966) and contained: 3.0 mM K2HPO4, 3.5 mM KH2PO4, 0.8 mM MgSO4, 0.04 mM MnCl2, 0.2 mM NaCl, 0.2 mM CaCl2, 0.05 mM ZnCl2, 0.04 mM FeCl3, 2 mM nitrogen source, 20 mM carbon source. Glutamic acid was

used as a nitrogen source when carbon sources were tested. The carbon sources investigated were: fructose, glucose, glutamic acid, glycerol, L-asparagine, L-glutamine, lactose, mannose, starch (5 g l^{-1}), sucrose, maltose and xylose. When nitrogen sources were tested, glucose and xylose were used as carbon source for ATCC 14579 and ATCC 10987 respectively. The nitrogen sources investigated were: NH4Cl, L-asparagine, L-glutamine, L-citrulline, L-isoleucine, L-serine, urea, Leu-Pro, Ala-Val, Gly-Gly and Ala-Tyr. The verification tests were performed in 1 ml minimal medium containing the desired nitrogen and carbon source in a 20 ml culture tube (Fisher Emergo, model 150 ¥ 17/18) and incubated up to 6 days at 30°C at 200 rpm (New Brunswick Scientific, model Innova 4335). When a culture showed visual turbidity, the strain was scored positive for the corresponding nutrient source.

Comparative genomics of ATCC 14579 and ATCC 10987

All predicted protein sequences of *B. cereus* ATCC 14579 (AE016877 and AE016878; Ivanova *et al*., 2003) and *B. cereus* ATCC 10987 (AE017194 and AE017195; Rasko *et al*., 2004) were downloaded from GenBank and saved as two separate FASTA formatted files. To reveal the strain-specific ORF, the two FASTA files were scanned against each other using the algorithm INPARANOID (default settings, BLOSUM45 scoring matrix; Remm *et al*., 2001). Unlike most bidirectional best hit-based methods INPARANOID has the advantage that it detects orthologues as well as in-paralogues or co-orthologues (duplicated genes after a recent speciation event). Hence, INPARANOID not only yields a single gene in one genome that is predicted to be the orthologue of a single gene in the other genome ('single to single'), but also yields 'many to single', 'single to many' and 'many to many' relationships. In the case of these in-paralogous relationships, it is not always apparent which of the duplicated genes retains the ancestral function (Notebaart *et al*., 2005). Therefore, orthologues as well as in-paralogues were subtracted from the lists of all genome ORFs to retrieve the strain-specific ORFs from each of the two *B. cereus* genomes. This resulted in two lists, containing the strain-specific ORFs. Clusters of Orthologous Groups were assigned to all strain-specific genes as described previously (von Mering *et al*., 2003; Tatusov *et al*., 2003) and the genes were listed with the corresponding annotations as used in the GenBank database. Subsequently, interrelating genome differences were searched for the observed phenotypic differences.

Results

Phenotypic comparison of ATCC 14579 and ATCC 10987

Utilization of carbon, nitrogen, sulphur and phosphorus sources and growth under several stress conditions, such as low and high pH, in the presence of salt and antibiotics were investigated with the Phenotypic Microarray (PM) system from Biolog. The results of the replicate experiments (2 × 1920 phenotypes tested) are displayed in Fig. 1 (the full PM report is included in Appendix S1). The data show that both strains can utilize numerous

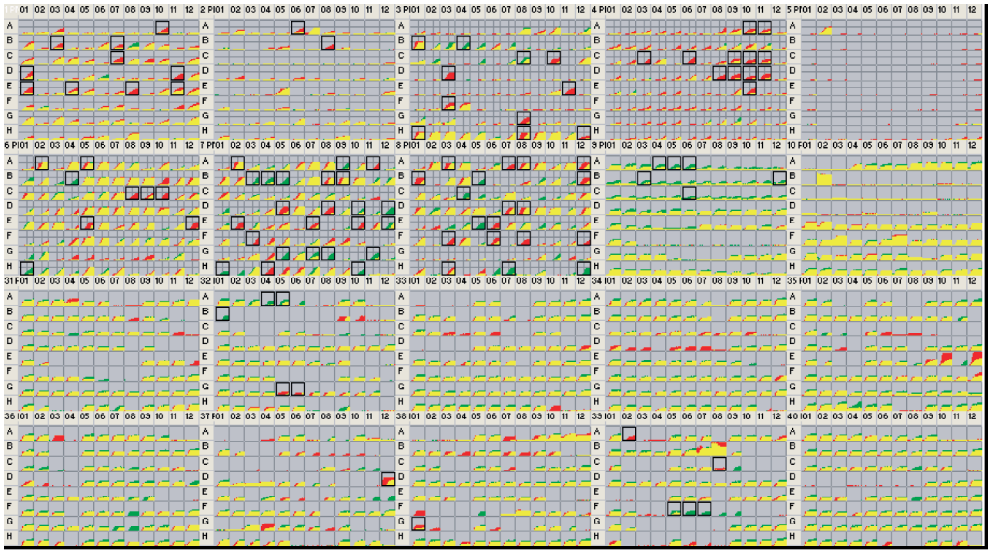


Fig. 1. Phenotypic Microarray analysis of 1920 phenotypes (20 PMs) of strains ATCC 14579 and ATCC 10987. The PM kinetic results show consensus data, comparing ATCC 14579 (red) and ATCC 10987 (green). Depicted are the twenty 96 well plates recorded, where each well corresponds with a tested substrate (http://www.biolog.com). Red indicates a stronger response by ATCC 14579, and green indicates a stronger response by ATCC 10987; when the two strains have equivalent metabolism or growth responses in a well, the red and green kinetic graphs overlap and are yellow. A box around a growth curve indicates a significant difference in response. The phenotypic differences are listed in Table 1 and for a detailed analysis see Appendix S1 in the *Supplementary material*.

nutrient sources and are resistant to several stresses and a range of chemicals and antibiotics. Also, ATCC 14579 has shown to grow on a larger number of carbon sources than ATCC 10987 in the PM analysis. Biolog recommends verification of the observed phenotypes of interest. Verification tests were set up to test the utilization of selected carbon and nitrogen sources. The resulting differences between ATCC 14579 and ATCC 10987, as determined by the PM and the verification tests are listed in Table 1. ATCC 14579 has shown to utilize several carbon sources, including a number of saccharides, asparagine, glutamine and glycerol, whereas ATCC 10987 showed no growth on these substrates. The PM revealed no phenotypic differences on carbon sources on which ATCC 10987 could grow and ATCC 14579 could not. However, the experiments in minimal medium showed that ATCC 14579 was not able to use xylose as a carbon source in contrast to ATCC 10987. The positive score on growth on xylose for ATCC 14579 in the PM appeared to be an artefact of xylose interacting with the dye used (M. Ziman, pers. comm., Biolog). The verification test confirmed the phenotypes for the substrates, trehalose, fructose, sucrose and glutamine. The phenotypic differences that were found for asparagine and glycerol utilization could not be confirmed in minimal medium: both strains were

unable to grow on asparagine and glycerol in minimal medium. Starch, a glucose polymer, has not been tested in the PM. In order to elucidate the glucose-based carbohydrate utilization by ATCC 14579, growth with starch as carbon and energy source has been tested in minimal medium and showed that only ATCC 14579 was able to grow on starch.
The observed phenotypes provide an overview of the metabolic capabilities of both strains. Figure 2 schematically describes the pathways used to metabolize several carbohydrates. ATCC 14579 can grow on several fructose and glucose-based carbohydrates whereas ATCC 10987 cannot. Maltose, however, is an exception. Both strains were capable of growing on maltose as the sole carbon source. The availability of other carbohydrates, such as ribose, gluconate and glucosamine, resulted in growth of both strains in the PM analysis. It has been suggested previously that ATCC 10987 can utilize tagatose (Rasko *et al*., 2004), but growth on tagatose was not observed in both the PM analysis and minimal medium.
The screening of nitrogen source utilization revealed large differences between both strains. *B. cereus* strain ATCC 14579 was able to utilize several amino acids and numerous dipeptides, especially aspartate and asparagine-containing dipeptides (Table 1). Growth of ATCC 14579 on Ala-Val was confirmed in minimal medium. The availability of Gly-Gly did not support growth of both strains in minimal medium, in contrast to the PM analysis, where ATCC 14579 showed growth on Gly-Gly. Other contradictory results, obtained in the verification tests, were growth of both strains on citrulline and glutamine, where the PM showed no growth of ATCC 10987. The dipeptide Leu-Pro could support growth of both strains according to the PM. However, in minimal medium only ATCC 14579 was able to utilize this dipeptide. Additionally, ammonium chloride (NH_4Cl) was tested as nitrogen source and revealed that only ATCC 14579 was capable of growing on this substrate. The PM indicated that strain ATCC 10987 was able to use isoleucine, D-serine and several, especially tyrosine-containing, dipeptides as sole nitrogen source for growth. The verification tests confirmed the phenotypic differences between the two strains on isoleucine and Ala-Tyr as nitrogen source.
Figure 3 shows a selection of pathways linked to nitrogen metabolism as derived from the observed phenotypes. Notably, both strains failed to grow on urea and nitrite as nitrogen sources, despite the presence of predicted urease and nitrite reductase genes. This scheme also reveals a selective advantage for ATCC 14579 as it is able to metabolize ammonium, glutamine and asparagine, whereas aspartate and glutamate are utilized by both strains.
The other categories of phenotypes included in the PM showed relatively few differences between the two strains. ATCC 14579 was able to use several phosphorus sources, including AMP, CMP and UMP isomers, whereas ATCC 10987 lacked this ability. Remarkably, the osmotolerance of ATCC 10987 (growth up to 5.5% w/v NaCl) was higher than that of ATCC 14579 (growth up to 3% w/v NaCl). The PM revealed a number of antibiotic resistance differences (Table 1). ATCC 14579 was more resistant to cell wall targeting antibiotics such as cefazolin, ketoprofen and moxalactam. ATCC 10987 has shown to be more resistant to antibiotics targeting protein synthesis such as chloramphenicol, thiamphenicol and lincomycin.

Table 1. Phenotypic differences between ATCC 14579 and ATCC 10987

Mode of action	Substrate(s)	Phenotype of ATCC 14579	Phenotype of ATCC 10987
C-Source	L-Glutamine[1], b-Methyl-D-Glucoside, Sucrose[1], D-Fructose[1], D-Trehalose[1], Glycerol[2], Dextrin, L-Asparagine[2], D;L-a-Glycerol-Phosphate, D-Fructose-6-Phosphate, 2`-Deoxy-Adenosine, Arbutin, Starch[3], D-Glucose[4]	+	-
	Xylose[4]	-	+
N-source	L-Pyroglutamic Acid, N-Acetyl-D-Glucosamine, L-Citrulline[2], g-Amino-N-Butyric Acid, Adenosine, L-Glutamine[2], Ammonium chloride[3]	+	-
	L-Isoleucine[1], D-Serine	-	+
Peptide N-source	Pro-Asp, Pro-Hyp, Gly-Asp, Asn-Val, Gly-Ser, Val-Asn, Ser-Gly, Lys-Asp,Gly-Asn, D-Ala-Gly, Asp-Glu, Gly-Gly[2], b-Ala-Gly, b-Ala-Phe, Val-Ala, Lys-Lys, Ala-Val[1], Ser-Asn, Ser-Asp, Ser-Val, Thr-Leu, Ser-Pro, Ala-Asp, Met-Gln, Asp-Asp, D-Leu-Tyr, Asp-Ala, Met-Asp, Ala-Asp, Met-Ala, Ala-Asn, Gly-Gly-Gly, Leu-Pro[4]	+	-
	Pro-Tyr, Tyr-Val, Tyr-Ala, Lys-Tyr, Met-Tyr, Tyr-Gly, Tyr-Lys, Lys-Trp, Ala-Tyr[1], Ile-Trp, His-His, Tyr-Glu, Lys-Ile, Val-Tyr, Thr-Arg, Lys-Thr, Tyr-Trp, Pro-Phe, Val-Tyr-Val, Tyr-Gly-Gly	-	+
P-source	Uridine-2';3'-Cyclic Monophosphate, Uridine-3'-Monophosphate, D-Glucosamine-6-Phosphate, Thymidine-5'-Monophosphate, Adenosine-5'-Monophosphate, Uridine-2'-Monophosphate, Cytidine-5'-Monophosphate, Cytidine-3'-Monophosphate, Cytidine-2';3'-Cyclic Monophosphate, Adenosine-2';3'-Cyclic-Monophosphate, Uridine-5'-Monophosphate, D-Glucose-1-Phosphate	+	-
Osmotic sensitivity	4% NaCl, 5% NaCl, 5.5% NaCl, 6% NaCl + L-Carnitine, 6% NaCl + N-N Dimethyl Glycine, 6% NaCl + Glutathione	-	+
Antibiotic resistance	Ketoprofen, Tunicamycin, Minocycline, Cefazolin, Moxalactam	+	-
	Thiamphenicol, Lincomycin, Chloramphenicol, t-Butyl Hydroquinone	-	+

[1] Phenotypic difference, as shown in PM, the difference was confirmed in minimal medium
[2] Phenotypic difference, as shown in PM, the difference was not confirmed in minimal medium
[3] Phenotypic difference, as shown in minimal medium, substance was not tested in PM analysis
[4] Phenotypic difference, as shown in minimal medium, the difference was not shown in PM analysis

Genome comparison of ATCC 14579 and ATCC 10987

The phenotypic differences found in this study could not be easily linked to data provided in previous genomics studies of the *B. cereus* group (Hoffmaster *et al*., 2004; Rasko *et al*., 2004; Han *et al*., 2006). Therefore, we performed a detailed comparative analysis of the *B. cereus* ATCC 14579 and ATCC 10987 genomes using INPARANOID. The 5.4 Mb chromosome of *B. cereus* ATCC 14579 contains 5234 predicted open reading frames (ORFs). These ORFs consisted of 6035 domains, of which 4223 (70%) could be assigned to a Cluster of Orthologous Groups (COG). pBClin15, the linear plasmid of ATCC 14579 is 15.3 kb and harbours 21 predicted ORFs. The chromosome of *B. cereus* ATCC 10987 is 5.2 Mb and contains 5603 predicted ORFs. These ORFs consisted of 6401 domains and a COG could be assigned to 4315 domains (67.4%). The plasmid of ATCC 10987, pBC10987, is 208.4 kb and harbours 240 predicted ORFs (Table 2).

Table 2. Genome comparison of *B. cereus* ATCC 14579 and ATCC 10987

	ATCC 14579			ATCC 10987		
	Chromosome	Strain specific	Plasmid	Chromosome	Strain specific	Plasmid
Size (kb)	5411.81		15.274	5224.28		208.369
GC %	35		38	35		33
ORFs	5234	983	21	5603	1360	240
Domains[1]	6035	1081	23	6401	1455	254
Domains with COG[2]	4223	436	5	4315	531	76
Domains without COG	1812	645	18	2086	924	178
Domains unknown function	376	35	0	385	36	8
Domains general function	634	67	0	654	90	6

[1] Note that one ORF can contain more than one domain that is or is not listed in a COG
[2] One domain can be listed in more than one COG

In the *Supplementary material* the complete set of strain-specific genes can be found (Table S1 and Table S2). Strain ATCC 14579 contains 983 genes without an orthologue in ATCC 10987. These genes consisted of 1081 domains, of which 645 (59.7%) were not assigned to a COG. Of the remaining 436 domains (40.3%), 35 were assigned to the function unknown group and for 67 domains only a general function was predicted, leaving 334 domains (30.9%) with a putative function description and COG. Strain ATCC 10987 contains 1360 genes with no orthologue in ATCC 14579. These genes consisted of 1455 domains, of which 924 (63.5%) were not in a COG. Of the remaining 531 domains (36.4%), 36 were assigned to the function unknown group and to 90 domains only a general function was assigned, resulting in 405 domains (27.8%) with a putative function description.

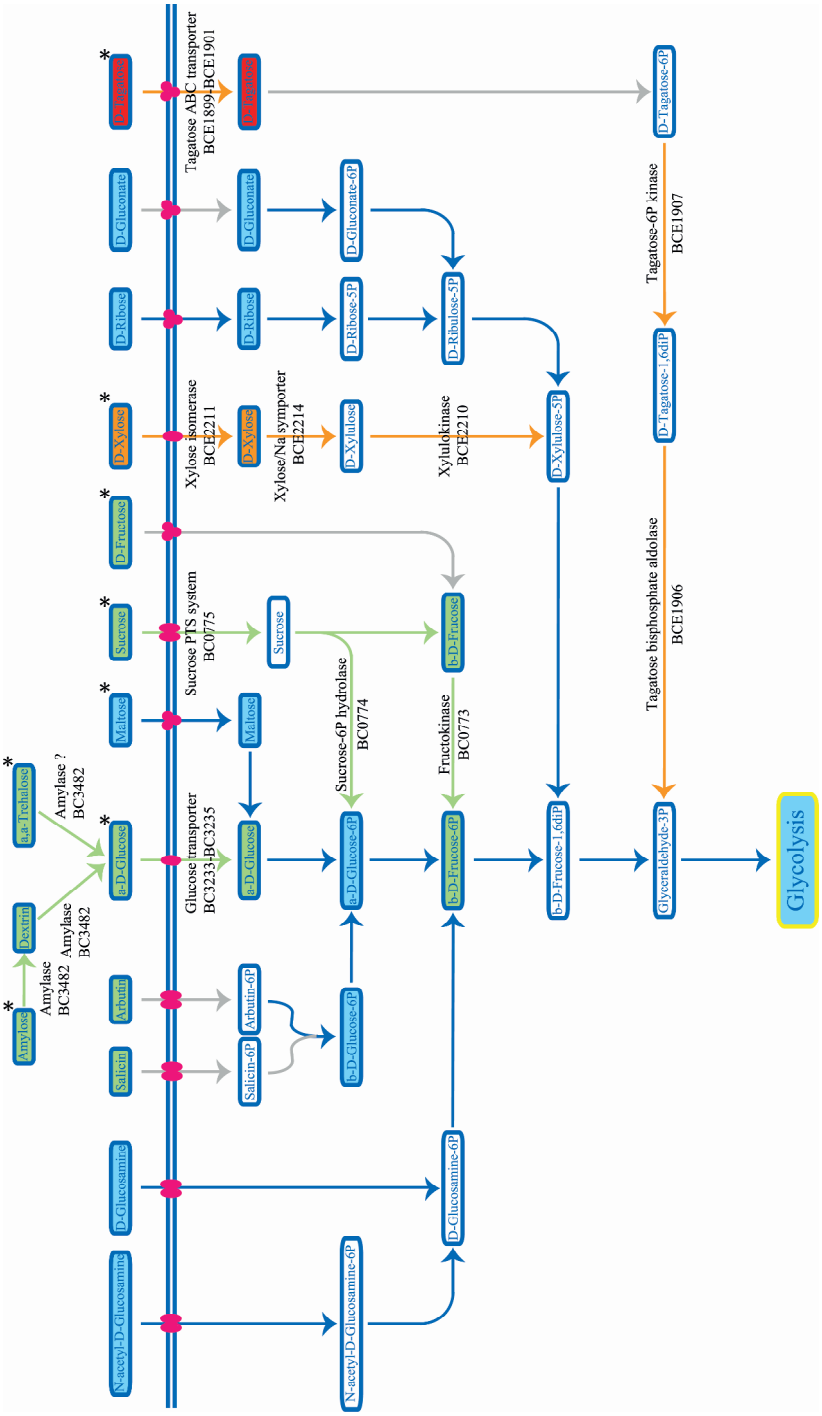

Amylose
Dextrin
a,a-Trehalose
Arbutin
Salicin
b-D-Glucosamine
N-acetyl-D-Glucosamine
a-D-Glucose
Maltose
Sucrose
D-Fructose
D-Xylose
D-Ribose
D-Gluconate
D-Tagatose
Amylase BC3482
Amylase BC3482
Amylase ? BC3482
Glucose transporter BC3233-BC3235
Sucrose PTS system BC0775
Xylose isomerase BCE2211
Xylose/Na symporter BCE2214
Tagatose ABC transporter BCE1899-BCE1901
Arbutin-6P
Salicin-6P
b-D-Glucose-6P
N-acetyl-D-Glucosamine-6P
D-Glucosamine-6P
a-D-Glucose-6P
Sucrose
Maltose
D-Xylose
D-Ribose
D-Gluconate
D-Gluconate-6P
D-Ribose-5P
D-Xylulose
D-Ribulose-5P
D-Xylulose-5P
Sucrose-6P hydrolase BC0774
Fructokinase BC0773
Xylulokinase BCE2210
b-D-Fructose
b-D-Fructose-6P
b-D-Fructose-1,6diP
Tagatose bisphosphate aldolase BCE1906
Tagatose-6P kinase BCE1907
D-Tagatose-6P
D-Tagatose-1,6diP
Glyceraldehyde-3P
Glycolysis

Fig. 2. Metabolic map of several carbohydrates utilized by ATCC 14579 and or ATCC 10987 with interrelating strain-specific genes. Green box, substance supports growth of ATCC 14579. Orange box, substance supports growth of ATCC 10987. Blue box, substance supports growth of both strains. Red box, substance did not support growth of both strains. White box, substance not tested for growth. Blue arrow, enzyme identified in both genomes. Grey arrow, enzyme not identified in both genomes. Green arrow, ATCC 14579 specific enzyme, shown with corresponding annotation and gene number. Orange arrow, ATCC 10987 specific enzyme, shown with corresponding annotation and gene number. Substances indicated with an asterisk were also investigated with minimal medium. The double blue line represents the bacterial membrane and is depicted with transporters (red ovals).

The strain-specific ORFs were scattered over the genome and were not confined to genomic islands (Fig. 4A). No large differences were observed in the distribution of the functional groups between ATCC 14579 (Fig. 4B) and ATCC 10987 (Fig. 4C).

Interrelation of genomes and phenotypes

Carbon sources. Phenotypic analysis revealed both *B. cereus* strains to grow on maltose. The disaccharide maltose is transported into the cell and subsequently hydrolysed inside the cell into two glucose residues. This suggests that the enzymes required for metabolizing glucose are present in both strains (Fig. 2). Therefore, the observed phenotypic difference concerning growth on glucose could be explained by a difference in glucose transport. The transport of glucose across the membrane can be facilitated by a glucose transporter, as present on the genome of ATCC 14579 (BC3233-BC3235) (Table 3). These genes could not be identified in the genome of ATCC 10987, which may explain this strain's inability to grow efficiently on glucose in our verification experiments (Table 1). The results of the PM on the other hand showed an insignificant difference in one of the runs, therefore this difference in glucose utilization was not noted. The reason for this insignificant difference remains unclear.

Amylose, a component of starch, and the disaccharide trehalose consist of glucose residues. In amylose, the glucose residues are linked in a 1→4 manner and in trehalose the residues are 1→1 linked. ATCC 14579 contains an ORF (*amyS*, BC3482) with COG0366, which is absent in ATCC 10987. This ORF is annotated as a glycosidase and contains a domain that is present in both amylases and trehalases. Glycosidases can break the glycoside linkages between glucose residues. Therefore, the presence of this glycosidase (BC3482), in combination with the glucose transporter, may support growth of *B. cereus* ATCC 14579 on trehalose and starch as indicated in Fig. 2 and Table 3.

Sucrose is transported into the cell and subsequently hydrolysed into glucose and fructose residues. Therefore, as for glucose, the phenotypic difference of growth on sucrose could be dependent on its transport. Sucrose can be transported into the cell via a phosphotransferase system (PTS). The genome of ATCC 14579 harbours the genes encoding such a system. In contrast, these PTS encoding genes could not be identified in the genome of ATCC 10987. In ATCC 14579, the sucrose PTS system is encoded by the following genes: BC0774,

BC0775 and BC0776. The presence of this transport system in ATCC4579 corresponds with the observed phenotypes (Table 3). Additionally, within the same strain-specific gene cluster, a fructokinase-encoding gene (BC0773) is located. Fructokinase converts fructose into fructose-6-phosphate. Fructose-6-phosphate can subsequently be used in glycolysis and in other metabolic pathways. ATCC 14579's ability to grow on fructose can be interrelated with the presence of this fructokinase (Fig. 2 and Table 3). The fructokinase could also facilitate the metabolism of fructose residues originating from sucrose.

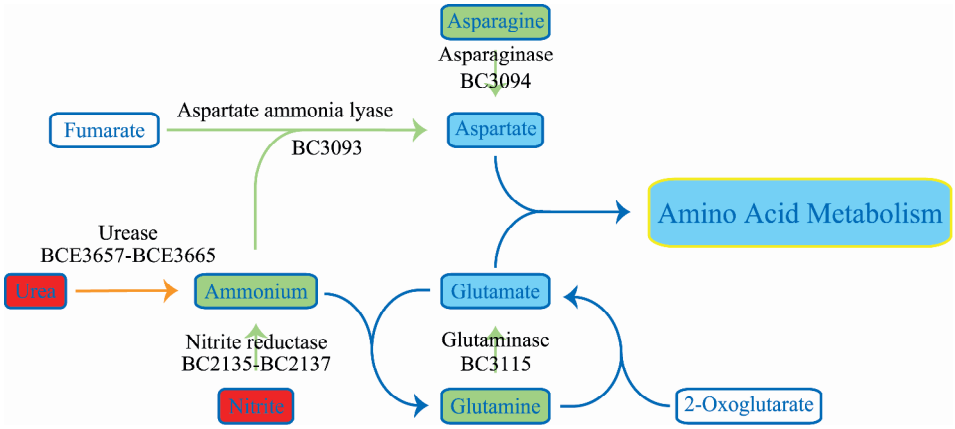


Fig. 3. Schematic representation of the metabolic routes of the nitrogen metabolism of *B. cereus*. Green box, substance supports growth of ATCC 14579. Orange box, substance supports growth of ATCC 10987. Blue box, substance supports growth of both strains. Red box, substance did not support growth of both strains. White box, substance not tested for growth. Blue arrow, enzyme identified in both genomes. Grey arrow, enzyme not identified in both genomes. Green arrow, ATCC 14579 specific enzyme, shown with corresponding annotation and gene number. Orange arrow, ATCC 10987 specific enzyme, shown with corresponding annotation and gene number.

Xylose can be imported into the cell and converted into xylulose-6-phosphate via several steps using xylose isomerase and xylulokinase (Fig. 2). In *Bacillus subtilis*, these enzymes are encoded on a specific gene cluster (Wilhelm and Hollenberg, 1984). ATCC 10987 harbours a homologous cluster on a gene replacement, previously described (Rasko *et al*., 2004). This ATCC 10987 specific gene cluster contains, besides a xylose isomerise (BCE2210) and a xylulokinase (BCE2211), a xylose repressor (BCE2208), an aldose epimerase (BCE2212) and a xylose transporter (BCE2214). These genes could facilitate the metabolism of xylose as described in Fig. 2. The phenotypic difference depicted corresponds with the lack of a xylose utilization cluster in the genome of ATCC 14579.
Figure 2 shows the metabolic pathway for the utilization of N-acetyl-glucosamine, glucosamine, maltose, gluconate and ribose. Both strains contain the genes encoding the

enzymes required for the metabolism of these substrates. Correspondingly, both strains were shown to use these substrates as sole carbon source (Appendix S1).

Nitrogen sources. At least two routes are known for the incorporation of ammonium (Fig. 3). Ammonium together with fumarate can be converted to aspartate and together with glutamate, glutamine is formed. Glutamine together with 2-oxoglutarate can be converted into two glutamate molecules. Subsequently, glutamate can be used as a substrate for amino acid metabolism. Only ATCC 14579 contains the genes required for these two routes. In addition aspartate ammonia-lyase (BC3093), catalysing the formation of aspartate from ammonium and fumarate, corresponds with the observed phenotype for ATCC 14579 displaying growth with ammonium as sole nitrogen source. Aspartate can also be formed from asparagine, which is converted into aspartate by the enzyme asparaginase. An asparaginase encoding gene can be found on the genome of ATCC 14579 next to aspartate ammonium-lyase. This asparaginase (BC3094) is specific for ATCC 14579 and corresponds with the observed phenotypes. On the same large gene replacement (partly described by Han *et al.*, 2006, Fig. S1) in the genome of ATCC 14579, a gene encoding glutaminase (BC3115) was identified. Glutaminase catalyses the conversion of glutamine into glutamate and ammonium. The presence of the glutaminase may explain the observed phenotypes. ATCC 14579 has shown to use glutamine both as nitrogen and carbon source (Fig. 1), while ATCC 10987 used glutamine only as a nitrogen source (Table 1). When carbon sources are sufficiently available, ATCC 10987 can produce glutamate from glutamine with 2-oxoglutarate. Without additional carbon sources, the glutaminase still enables ATCC 14579 to produce glutamate. The presence of another ATCC 14579 specific gene of this cluster, glutamine permease (BC3116), did not show a phenotypic effect, as indicated by the ability of ATCC 10987 to use glutamine as a nitrogen source.

The genes *azlC* and *azlD* present in the genome of ATCC 10987 (BCE4092 and BCE4093 respectively), putatively encode Branch Chained Amino Acid (BCAA) transporters. Correspondingly, ATCC 10987 was able to use isoleucine as nitrogen source (Table 1). The absence of these genes in ATCC 14579 corresponds with the observation that this strain failed to use isoleucine as a nitrogen source.

Dipeptides. Many differences between the two strains were found in dipeptide utilization (Table 1). In general, ATCC 14579 grows on dipeptides containing negatively charged or polar residues (glutamine, glutamate, asparagine and aspartate). On the other hand, ATCC 10987 is able to utilize polar and tyrosine containing dipeptides. Dipeptides are transported by Opp (oligo peptide) like transport systems, with OppA as the substrate binding component. ATCC 10987 harbours two additional *oppA* copies (BCE0247, BCE1917) and a complete additional *opp* system (BCE0724-BCE0728) in comparison with ATCC 14579. The additional OppA's and the Opp system together may mediate the transport of other dipeptides. This could lead to the observed phenotypic differences, with each strain utilizing specific dipeptides.

Table 3. Differences in nutrient utilization of ATCC 14579 and ATCC 10987 and putative interrelating gene (cluster).

C-source	Putative interrelating gene (cluster) and function Annotation of gene (cluster) as present in ATCC 14579	Corresponding gene number(s)
Trehalose	Glycosidase *amyS*	BC3482
Fructose	Fructokinase *ydjE*	BC0773
Sucrose	Sucrose PTS cluster	BC0774-BC0776
L-glutamine	Glutaminase *ybgJ*, Glutamine permease *ybgH*	BC3115-BC3116
Starch	Glycosidase *amyS*	BC3482
Glucose	Glucose/ mannose transporter	BC3233-BC3235
	Annotation of gene (cluster) as present in ATCC 10987	
Xylose	Xylose utilisation cluster	BCE2208, BCE2210-BCE2212, BCE2214
Tagatose[1]	Tagatose utilization cluster	BCE1899-BCE2008

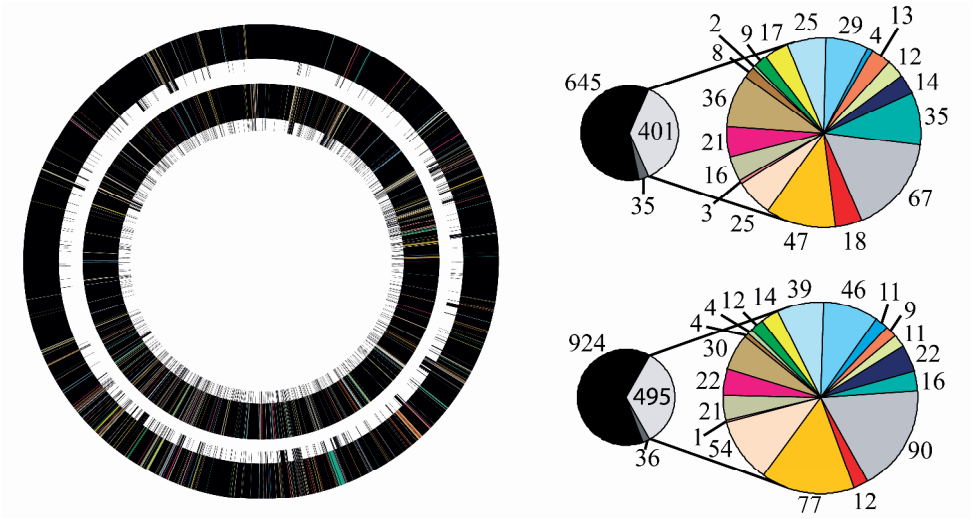
N-source	Annotation of gene (cluster) as present in ATCC 14579	Corresponding gene number(s)
NH_4Cl	Aspartate ammonia-lyase	BC3093
Leu-Pro	Proline iminopeptidase	BC2337
L-glutamine	Glutaminase *ybgJ*, Glutamine permease *ybgH*	BC3115-BC3116
	Annotation of gene (cluster) as present in ATCC 10987	
L-isoleucine	BCAA transport *azlC*, *azlD*	BCE4092-BCE4093
Urea[1]	Urease cluster	BCE3657-BCE3664

[1] Phenotypic difference suggested based on the presence of interrelating gene cluster, however no phenotypic difference observed.

Besides the various peptide transport systems that mediate the uptake of peptides, also peptidases, which facilitate peptide cleavage, are poorly characterized. The *B. cereus* genome harbours numerous putative peptidases, but their substrate specificity remains largely unclear. However, one of the better characterized peptidases is proline iminopeptidase that can cleave proline-containing dipeptides. The verification experiments showed that only ATCC 14579 could utilize the Leu-Pro dipeptide (Table 1). On the genome of ATCC 14579 a strain-specific intercellular proline iminopeptidase (BC2337) could be identified. Therefore, it is plausible that ATCC 14579 is capable of cleaving the Leu-Pro dipeptide and able to metabolize the corresponding amino acids to support growth (Table 3). ATCC 10987 does not harbour this gene and this corresponds with the lack of growth shown for this strain on Leu-Pro.

Phosphorus sources. The PM results showed that ATCC 14579 was able to use UMP, AMP, CMP and TMP (monophosphate derivatives of uridine, adenosine, cytidine and thymidine respectively), whereas ATCC 10987 was only able to use GMP (Table 1). Inosine-monophosphate (IMP) can be converted into GMP via xanthine conversions. This means that GMP can be synthesized via UMP, PRPP (5-phosphoribosyl diphosphate) and IMP. The xanthine metabolism genes were present as an insertion on the genome of ATCC 14579 (BC3164-BC3166). Via this pathway ATCC 14579 can synthesize GMP from the other nucleosides. ATCC 10987 lacks this pathway and lacks the ability to use other nucleosides than GMP. The interlinking of the xanthine pathway with the phenotypes

observed was confirmed by the growth of ATCC 10987 on GMP, because the xanthine pathway is not required to convert GMP into the other nucleosides.



	ATCC 14579		ATCC 10987			
Colour	#	%	#	%	COG code	COG group
	18	1.67	12	0.82	J	Translation
	0	0	0	0	A	RNA processing and modification
	47	4.35	77	5.29	K	Transcription
	25	2.31	54	3.71	L	Replication, recombination and repair
	0	0	0	0	B	Chromatin structure and dynamics
	3	0.28	1	0.07	D	Cell cycle control, mitosis and meiosis
	0	0	0	0	Y	Nuclear structure
	16	1.48	21	1.44	V	Defense mechanisms
	21	1.94	22	1.51	T	Signal transduction mechanism
	36	3.33	30	2.06	M	Cell wall/ membrane biogenesis
	8	0.74	4	0.27	N	Cell motility
	0	0	0	0	Z	Cytoskeleton
	0	0	0	0	W	Extracellular structures
	2	0.19	4	0.27	U	Intracellular trafficking and secretion
	9	0.83	12	0.82	O	Posttranslational modification, protein turn-over, chaperones
	17	1.57	14	0.96	C	Energy production and conversion
	25	2.31	39	2.68	G	Carbohydrate transport and metabolism
	29	2.68	46	3.16	E	Amino acid transport and metabolism
	4	0.37	11	0.76	F	Nucleotide transport and metabolism
	13	1.20	9	0.62	H	Coenzyme transport and metabolism
	12	1.11	11	0.76	I	Lipid transport and metabolism
	14	1.30	22	1.51	P	Inorganic ion transport and metabolism
	35	3.24	16	1.10	Q	Secondary metabolites biosynthesis, transport and catabolism
	67	6.20	90	6.19	R	General function prediction only
	35	3.24	36	2.47	S	Function unknown
	645	59.67	924	63.51	*	Not in COGs

Fig. 4. Genomic differences between ATCC 14579 and ATCC 10987. A. The inner ring represents the chromosome of ATCC 10987. The chromosome of ATCC 14579 is represented by the outer ring. All genomic differences are located on their relative distance from the origin of replication of the chromosome (top). Every colour corresponds with a COG group as listed in D. B. ATCC 14579 specific domains clustered based on COG groups, colours and numbers correspond with D. C. ATCC 10987 specific domains clustered based on COG groups, colours and numbers correspond with D. D. Each colour used in this figure represents a COG group (last column) with a corresponding COG code. For both strains the number of strain-specific domains per COG group (#) and the percentage of a COG group, within all genome differences, are indicated.

Nitrate respiration, urease and glutamate decarboxylase and haemolysin. The presence of nitrite reductase genes (BC2136-BC2138) and nitrate reductase genes (BC2118-BC2121) may contribute to several processes in *B. cereus*. Nitrate reductase can convert nitrate into nitrite and nitrite reductase can convert nitrite into ammonium (Fig. 3). The ability of ATCC 14579 to use NH_4^+ as a nitrogen source (Fig. 3) suggests that this strain can use its precursors nitrate and nitrite as nitrogen sources. However, this was not observed in the PM analysis (Fig. 1). Nitrate and nitrite reductases may also function in an alternative electron transport chain to support growth under oxygen-limited conditions. Nitrite and nitrate were not essential for growth of these strains under anaerobic conditions (data not shown, and Rosenfeld *et al*., 2005). However, an increased yield can be observed when ATCC 14579, but not ATCC 10987, is grown anaerobically in LB medium supplemented with nitrate (data not shown). Consequently, a phenotype could be related to the occurrence of nitrate (and nitrite) reductase in ATCC 14579.

Urea may act as a nitrogen source supporting bacterial growth. Urea can be degraded by urease to carbon dioxide and ammonia. An insertion in the genome of ATCC 10987, described by Rasko and colleagues (2004), contains genes encoding the subunits of urease (BCE3657-BCE3659) and other facilitating proteins (BCE3660-BCE3665). Although, ATCC 10987 contains this urease cluster, growth with urea as the sole nitrogen source was not observed (Table 3). Furthermore, the urease negative strain ATCC 14579 was just as resistant to urea as ATCC 10987 (Fig. 1, PM9 E7 to E12). Rasko and colleagues (2004) suggested an enhanced acid stress survival of ATCC 10987, due to the presence of the urease cluster. However, the PM showed no difference in growth under acidic conditions between the two strains (Fig. 1, PM10 A1 to A12). It is conceivable that the presence of a glutamate decarboxylase (BCE2691) also does not contribute to the growth of *B. cereus* ATCC 10987 in acidic environments (Fig. 1), because a gene encoding a glutamate/GABA (gamma-amino butyric acid) antiporter is lacking. As previously observed for *Listeria monocytogenes* (Cotter *et al*., 2001), acid tolerance is only provided when the glutamate decarboxylase acts in concert with a glutamate/GABA antiporter thereby maintaining intracellular pH homeostasis by removing intracellular protons (Cotter *et al*., 2001).

The genomic island of ATCC 14579, on which glutaminase is present, harbours genes encoding the haemolytic enterotoxin Haemolysin BL (HBL; Beecher and Macmillan, 1991). This enterotoxin consists of three components; HBL binding component precursor

(present in duplicate, BC3101 and BC3102), HBL lytic component L1 and HBL lytic component L2. All genes encoding these HBL enterotoxin components were absent in ATCC 10987. This correlates with the inability of ATCC 10987 to produce HBL, while ATCC 14579 is capable of producing this haemolytic enterotoxin (Hansen and Hendriksen, 2001).

Discussion

This study shows that comparative genomics combined with an extensive phenotypic characterization of *Bacillus cereus* strains ATCC 14579 and ATCC 10987 provides detailed insight in their growth performances and metabolic capacities. We identified numerous strain-specific ORFs that were not identified in previous studies (Rasko *et al*., 2004). Han and colleagues (2006) describe that *B. cereus* ATCC 14579 contains a 17.7 kb large insert that is lacking in ATCC 10987. Close analysis of the INPARANOID output shows that this insertion is much larger (40.6 kb), and that the extended insert contains, among others, genes encoding aspariginase and glutaminase, enzymes involved in ammonium utilization and nitrogen metabolism (Fig. S1). Moreover, the role of plasmids as information carriers within the *B. cereus* group has been discussed previously and it was suggested that the main differences between strains, such as the main virulence factors, are contained on plasmids (Han *et al*., 2006; Rasko *et al*., 2007). However, we found a large number of phenotypic differences between the two strains studied, which could be attributed to chromosomal genes but not to genes encoded on plasmids. This suggests that a more extensive comparative genotypic and phenotypic analysis may reveal more differences between species and strains within the *B. cereus* group than previously assumed.

It has previously been suggested (Ivanova *et al*., 2003) that *B. cereus* shows a prevalence for the metabolism of proteins, peptides and amino acids over the metabolism of carbohydrates. Here we provide evidence that *B. cereus* ATCC 14579 can also metabolize a large number of carbohydrates besides utilizing a broad range of peptides and amino acids for growth. As carbohydrates and nitrogen compounds are important food constituents, the metabolism of these compounds can contribute to the performance of *B. cereus* ATCC 14579 in food. *Bacillus cereus* ATCC 10987 was isolated from a spoiled cheddar cheese in Canada (Rasko *et al*., 2004). This suggests that this strain can cope efficiently with high salt-containing environments, because NaCl concentrations in cheddar cheeses may range from 1.33% to 1.83% NaCl (w/w in total cheese) (Nair *et al*., 2004). Phenotypic Microarray analysis revealed *B. cereus* strains ATCC 10987 and ATCC 14579 to be able to grow up to 5% and 3.5% NaCl (w/v) respectively. No strain-specific genomic features were found that could be directly linked to osmo-tolerance, e.g., typical osmolyte transporter genes were found on both genomes. However, an additional extracytoplasmic function (ECF) sigma factor was identified on the genome of *B. cereus* ATCC 10987 that shows similarity with an ECF sigma factor involved in the stress response of *Listeria monocytogenes* (Zhang *et al*., 2005). Whether this regulator can be related to the increased osmo-tolerance of *B.*

cereus ATCC 10987 needs to be investigated in more detail. The growth performance in acidic conditions appeared to be similar for both strains. This indicates that under these specific conditions the urease cluster (Rasko *et al*., 2004) and the glutamate decarboxylase do not supply growth advantage to *B. cereus* ATCC 10987 in acidic environments. Such an advantage may be displayed under conditions not tested here, and the role of these genes in, for example, acid shock conditions remains to be established. *Bacillus cereus* ATCC 14579 was isolated from the air in a cow shed (Frankland and Frankland, 1887). The environment, e.g., soil, silage, insect or mammalian gastrointestinal tract, from which ATCC 14579 originates is unknown. However, its ability to utilize several glucose and fructose-based carbohydrates could supply a growth advantage for this strain in starchy foods such as rice and pasta.

Our results support the opinion expressed by White (2006), stating that biochemical and phenotypic investigations are needed to find functions for hypothetical genes, and that *in silico* suggested performances need to be confirmed experimentally. A broad screening like the PM analysis can be useful for such studies. As indicated by Biolog, the phenotypes of interest observed with the PM technique should be verified. In interpreting the differences between the Biolog results and the verification tests it should be recognized that the conditions are different (medium composition, oxygen availability, incubation period). Therefore, it cannot be concluded that one technique is superior over the other. Both experiments have their advantages and disadvantages, for instance the PM enables a very broad screening in a single experiment, the tests in minimal medium on the other hand are chemically defined. In combination these experiments help in advancing our understanding of the growth performance of *B. cereus.*

The approach used, by establishing phenotypic differences independent of genetic variety, gives indications where to expect differences which could be missed in a conventional approach. For example, the glucose transporter genes could easily be missed, due to the abundance of transporters and the presence of all other genes necessary for glucose metabolism, demonstrated by the successful growth on maltose by both strains. Second, the differences in peptide metabolism could not have been discovered using comparative genomics with the present state of annotation, because the substrate specificity of most peptide transporters has not been established. Therefore, interlinking genetic information, contained in genome sequences, and corresponding phenotypes is a promising approach to assess the growth performance and metabolic capacity of microorganisms. The approach used can supply indicators for the performance of these bacteria in different environments and ecological niches.

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Supplementary material
The following supplementary material is available for this chapter online (http://www.blackwell-synergy.com):
Fig. S1. *B. cereus* ATCC 14579 asparaginase and glutaminase region. The 40.6 kb gene replacement of strain ATCC 14579 compared with the corresponding 9.8 kb region on the genome of ATCC 10987. The glutaminase, asparaginase and other amino acid metabolism-associated genes are shown in light blue. Other gene clusters are indicated, such as sporulation and germination-related genes (red) and haemolytic enterotoxin genes (yellow). The gene numbers are depicted in the corresponding arrow, gene names are listed underneath or above the corresponding arrow. The first and the last gene are orthologous in both genomes.
Table S1. *Bacillus cereus* strain ATCC 14579 specific ORFs.
Table S2. *Bacillus cereus* strain ATCC 10987 specific ORFs.
Appendix S1. Phenotype Microarray report from Biolog.

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Chapter 3

Role of ureolytic activity in *Bacillus cereus* nitrogen metabolism and acid survival

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Abstract
The presence and activities of urease genes were investigated in 49 clinical, food, and environmental *Bacillus cereus* isolates. Ten strains were shown to have urease genes, with eight of these strains showing growth on urea as the sole nitrogen source. Two of the urease-positive strains, including the sequenced strain ATCC 10987, could not use urea for growth, despite their capacities to produce active urease. These observations can be explained by the inability of the two strains to use ammonium as a nitrogen source. The impact of urea hydrolysis on acid stress resistance was subsequently assessed among the ureolytic *B. cereus* strains. However, none of the strains displayed increased fitness under acidic conditions or showed enhanced acid shock survival in the presence of urea. Expression analysis of urease genes in *B. cereus* ATCC 10987 revealed a low level of expression of these genes and a lack of pH-, nitrogen-, urea-, oxygen-, and growth phase-dependent modulation of mRNA transcription. This is in agreement with the low urease activity observed in strain ATCC 10987 and the other nine strains tested. Although a role for *B. cereus* ureolytic activity in acid survival cannot be excluded, its main role appears to be in nitrogen metabolism, where ammonium may be provided to the cells in nitrogen-limited, urea-containing environments.

Introduction

Bacillus cereus is a Gram-positive, spore-forming rod known to be present in various environments. Vegetative cells and/or spores can be found in soil (Vilain *et al.*, 2006), rhizospheres (Berg *et al.*, 2005), air (Frankland and Frankland, 1887), insect guts (Luxananil *et al.*, 2001), and foods (Kotiranta *et al.*, 2000; Rosenquist *et al.*, 2005) and in the human gastrointestinal tract (Ghosh, 1978). *B. cereus* can cause a diarrheal and an emetic type of food-borne disease. The emetic type of disease occurs when *B. cereus* cells grow and produce the heat-stable emetic toxin cereulide in food. The emetic symptoms arise after ingestion of intoxicated food (Turnbull, 1981; Agata *et al.*, 2002). The diarrheal type of disease is caused upon ingestion of *B. cereus* vegetative cells and/or spores and their survival of passage through the stomach, followed by germination and/or growth in the human small intestine, where vegetative cells produce several enterotoxins (Turnbull, 1981; Granum and Lund, 1997; Wijnands *et al.*, 2007).

B. cereus can encounter suboptimal conditions for growth in various environments. The presence and activity of alternative pathways to metabolize nitrogen sources, such as urease-mediated degradation of urea, can give bacteria a growth advantage under nitrogen-limited conditions. In addition, the bacteria may be exposed to stress conditions and display adaptive stress responses to cope with suboptimal conditions, such as low pH. For instance, Gram-positive bacteria may activate decarboxylases, deaminases, proton pumps, and urease to cope with acid stress (Cotter and Hill, 2003).

Urease (EC 3.5.1.5) catalyzes the hydrolysis of urea, generating two molecules of ammonia and one molecule of carbon dioxide, and it was the first enzyme to be crystallized (Sumner, 1926). Urease is present in various organisms, including plants, fungi, and bacteria. Urease activity has been associated with various diseases, such as infection-induced urinary stones and peptic ulceration (Mobley and Hausinger, 1989). The role of urease in the pathogenesis of *Helicobacter pylori*, which causes peptic ulceration (Lee *et al.*, 1993), is evident. Ammonia molecules, produced upon hydrolysis of urea, can bind protons and consequently elevate the pH. This mechanism has been shown to be essential for the colonization of the human stomach by *H. pylori* (Tsuda *et al.*, 1994). Another role of urea hydrolysis is to provide ammonium for nitrogen metabolism, as has been reported for a range of bacterial species (Jahns *et al.*, 1988). A dual role of urease, in acid resistance and in nitrogen metabolism, for example, has been reported for *Streptococcus salivarius* (Chen *et al.*, 2000), *H. pylori* (Williams *et al.*, 1996), and *Yersinia enterocolitica* (Young *et al.*, 1996).

Rasko and colleagues (2004) identified a urease utilization cluster composed of nine genes (BCE3656 to BCE3664) in the genome of *B. cereus* strain ATCC 10987 that is not present in other sequenced strains belonging to the *B. cereus* group. This cluster harbours three genes, *ureA*, *ureB*, and *ureC*, encoding the structural enzyme that, respectively, show similarities of 62%, 44%, and 65% to the structural urease genes of *Bacillus subtilis* (Cruz-Ramos *et al.*, 1997). Besides the structural genes, the cluster harbours genes encoding accessory proteins (*ureE*, *ureF*, *ureG*, and *ureD*), which are required to incorporate nickel ions into the enzyme and to activate the enzyme (Soriano *et al.*, 2000). Furthermore, the

urease cluster of *B. cereus* ATCC 10987 contains two additional genes for a putative urea (acetamide) transporter (*ureI*) and a nickel transporter (*nikT*).
Urea is present in various environments in which *B. cereus* can be found, including soil, food, and the human host, where urea is present in all body fluids and is finally excreted in the urine (0.4 M to 0.5 M urea) as a detoxification product (Mobley and Hausinger, 1989). *B. cereus* encounters urea upon its interaction with the human host: human saliva contains 2.3 mM to 4.1 mM (Dawes and Dibdin, 2001), the human stomach contains approximately 4.8 mM (Neithercut *et al.*, 1993), and the urea concentration in human blood varies from 1.7 mM to 8.3 mM (Mackay and Mackay, 1927). Urea can also be present in various foods. Foods of animal origin generally contain urea; for example, milk contains 4.4 mM to 6.4 mM urea (Carlsson *et al.*, 1995). Although urease may play an important role in the life cycle of *B. cereus*, information is lacking about its role in nitrogen metabolism and acid survival in this human pathogen.
In this study, we investigated the prevalence of urease genes and urease activity among 49 environmental, food, and clinical *B. cereus* isolates. Furthermore, urease gene expression and the roles of ureolytic activity in nitrogen metabolism and in acid resistance were assessed.

Materials and methods

Bacterial strains and culture conditions
B. cereus strains from various sources, including clinical, environmental, and food isolates, together with the type strain (ATCC 14579) and other sequenced strains, such as ATCC 10987 and PAL25 (also known as AH187 (Rasko *et al.*, 2007) and F4810/72 (Haggblom *et al.*, 2002)), were used in this study (see Table 2). A number of strains were kindly provided by P. E. Granum (Norwegian School of Veterinary Science, Oslo, Norway), NIZO Food Research (Ede, The Netherlands), Institut National de la Recherche Agronomique (Avignon, France), and the National Institute for Public Health and the Environment (Bilthoven, The Netherlands). Stock cultures grown in brain heart infusion (BHI) (Becton Dickinson, France) broth were stored at -80°C in 50% glycerol. To prepare aerobic working cultures, 10 ml BHI in a 100-ml Erlenmeyer flask was inoculated with a droplet from the glycerol stock and incubated overnight at 30°C with shaking at 200 rpm. Oxygen-limited working cultures were prepared in 50 ml BHI in a completely filled 50-ml tube (Greiner Bio-one, Germany) and incubated overnight at 30°C.

PCR detection of urease genes
The primers used in the PCR (listed in Table 1) were designed from the *ureA* (BCE3662) and *ureC* (BCE3664) genes from *B. cereus* ATCC 10987 (accession number AE017194) (34). *Taq* DNA polymerase (MBI Fermentas, Germany) was used to amplify the target genes. The PCR was performed in an MJ Research PTC-200 thermal cycler preset to 30 cycles of 15 seconds at 94°C, 15 seconds at 45°C, and 1 min at 72°C. Template DNA was

obtained from 1 ml aerobic working culture, which was boiled two times for 1 min each time and put on ice after each boiling step.

Assessment of nutrient utilization

The utilization of glutamate, ammonium, and urea as sole nitrogen sources was tested in minimal medium. This medium was derived from GGGS medium (Buono *et al.*, 1966) and contained 3.0 mM K_2HPO_4, 3.5 mM KH_2PO_4, 0.8 mM $MgSO_4$, 0.04 mM $MnCl_2$, 0.2 mM NaCl, 0.2 mM $CaCl_2$, 0.05 mM $ZnCl_2$, 0.04 mM $FeCl_3$, 20 mM maltose, and 2 mM glutamate, ammonium, or urea (Merck, Germany, and Sigma, St. Louis, MO). One millilitre of aerobic working culture was spun down and washed with minimal medium without nitrogen sources. Subsequently, 200 µl of minimal medium supplemented with the desired nitrogen source was inoculated (1:100; initial optical density at 600 nm [OD600], 0.005-0.003) with the washed culture in a microtiter plate (Greiner Bio-one, Germany). The plate was incubated for up to 6 days at 30°C. The OD600 was measured each day (uQuant; Bio-Tek Instruments). The result was scored positive if the final OD was 0.05 or higher.

Urease activity assay

The urease activities of the strains that scored positive in the PCR test (Table 2) were tested as follows. One millilitre of oxygen-limited working culture in BHI was spun down and resuspended in 200 µl physiological salt solution (0.85% [w/v] NaCl in water), and a urease activity diagnostic tablet (Rosco Diagnostica, Denmark) containing urea and a pH indicator was added to the suspension. The suspension was incubated for 24 h at 30°C. The mixture changed colour, from yellow (low pH) to purple (high pH), upon the increase of pH caused by the urease activity. The urease activities of *B. cereus* strains ATCC 10987 and ATCC 14579 (negative control) were also monitored in MES [2-(*N*-morpholino)ethanesulfonic acid] (Sigma, Germany) and MOPS [3-(*N*-morpholino)propanesulfonic acid] (Sigma, Germany) buffers. Twenty millilitres of oxygen-limited working culture was spun down and resuspended in 4 ml 10 mM MES or 10 mM MOPS buffer with 10 mM urea at pH 5.2 and pH 6.5, respectively. The pH was measured over time with a PHM 240 pH/ION meter (Radiometer, Denmark). To assess the amount of ammonia formed in the urease assay, MES, pH 5.0, including ATCC 10987 cells was titrated to pH 9.0 with 100 mM NH_3 solution. The protein concentration of the aerobic working culture was determined by using the bicinchoninic acid assay to determine the urease enzyme activity of ATCC 10987 cells.

Acid growth assay and urea concentration measurements

To assess the growth performance of the *B. cereus* strains under acid conditions, 200 µl BHI, BHI supplemented with 10 mM urea, and BHI supplemented with 10 mM urea and 10 µM the urease inhibitor flurofamide (Tocris Biosciences, United Kingdom) at pH 7 and at pH 5 were inoculated with 2 µl aerobic *B. cereus* working cultures. The growth performance was assessed in microtiter plates (Greiner Bio-one, Germany) and with incubation for 10 h at 30°C. Every hour, the absorbance (600 nm) was measured (uQuant;

Bio-Tek Instruments). The growth of strains ATCC 10987 and ATCC 14579 was also tested in nutrient broth (NB) (Oxoid, England) at pH 7 and pH 5 with and without 10 mM added urea. The media used were acidified with HCl (37%; Merck, Germany) to pH 5.0. The pH was monitored with a PHM 240 pH/ION meter (Radiometer, Denmark). To quantify the endogenous urea concentration in BHI and NB, the Quantichrom urea assay kit (Bioassay Systems) was used according to the provided protocol.

Table 1. PCR and RT-RT-PCR primers used in this study

Primer name	Gene(s)	Gene ID(s)	Sequence
PCR			
UreaseForw	*ureABC*	BCE3662-BCE3664	ATCAGATATTCAAGTCGAGG
UreaseRev	*ureABC*	BCE3662-BCE3664	CCAGGTGTTATTGTAGTTGC
RT-RT-PCR			
QPCR_ureA_Forw	*ureA*	BCE3664	TTCCGCCACATTTTTACCATC
QPCR_ureA_Rev	*ureA*	BCE3664	ACGAAAGGAGAGGGGGCTTA
QPCR_ureG_Forw	*urge*	BCE3659	GCCCCTACATAAGGTGCCAAA
QPCR_ureG_Rev	*urge*	BCE3659	GCGCAGGGAGAAAAGATTCC
QPCR_ureI_Forw	*ureI*	BCE3657	GGAATGCCCAAAAGAACCAA
QPCR_ureI_Rev	*ureI*	BCE3657	CATTGCAACGGTTATGGGAAT
QPCR_nikT_Forw	*nikT*	BCE3656	CCATGTTTTGCCGTTTTTCC
QPCR_nikT_Rev	*nikT*	BCE3656	ACTCACAGGGGGTGGGATTT
QPCR_tufA_Forw	*tufA*	BCE0108	GCCCAGGTCACGCTGACTAT
QPCR_tufA_Rev	*tufA*	BCE0108	TCACGTGTTTGAGGCATTGG
QPCR_rpoA_Forw	*rpoA*	BCE0137	ACCGCTTGAGCGTGTGGATATG
QPCR_rpoA_Rev	*rpoA*	BCE0137	TAGCAGTAACAGCGGCACCA

Acid shock survival assay

The impact of urea on the acid shock survival capacities of the *B. cereus* strains was assessed with stationary-phase cells in 20 ml BHI, in 20 ml BHI with 10 mM added urea, and in 20 ml BHI with 10 mM added urea and 10 µM flurofamide. The cultures were inoculated with 100 µl aerobic working cultures and were subsequently incubated at 30°C with aeration (200 rpm). After overnight incubation, a preset amount of HCl was added to reach the desired acidic pH values. At designated time points (0, 5, 10, 20, and 30 min), dilutions (10^{-1}, 10^{-3}, and 10^{-5}) were made in peptone physiological salt solution (1 g l^{-1} neutralized bacteriological peptone [Oxoid, England] and 8.5 g l^{-1} NaCl in water). The survival of *B. cereus* was determined by droplet plating (Neblett, 1976), with 5 µl of the cultures and the corresponding dilutions put on BHI agar plates (15 g l^{-1} agar bacteriological; Oxoid, England) and incubated overnight at 30°C.

RNA isolation and RT-RT-PCR

B. cereus strain ATCC 10987, with the highest ureolytic activity in the urease activity assay, was used in the real-time reverse transcriptase (RT-RT) PCR analysis. To obtain RNA samples, 50 ml of oxygen-limited working culture was spun down and resuspended in

10 ml 10 mM MES or 10 mM MOPS buffer with 10 mM urea at pH 5.2 and pH 6.5, respectively. RNA isolation was performed by transferring 10 ml of the cultures at 0, 30, 90, and 180 min into a 50-ml Falcon tube, and the cultures were spun down at 13,000 *g* for 30 seconds. After the supernatant was decanted, the cell pellets were snap-frozen in liquid nitrogen. Within 20 min after the cell pellets were frozen, TRI-reagent (Ambion, United Kingdom) was added to the pellets and RNA was extracted as described previously (van Schaik *et al.*, 2004). The expression ratios of four genes from the urease cluster (*ureA*, *ureG*, *ureI*, and *nikT*) were determined using the oligonucleotides listed in Table 1. cDNA synthesis and RT-RT-PCR were performed as described previously, with *tufA* and *rpoA* as reference genes (van Schaik *et al.*, 2005). All RT-RT-PCRs were performed in duplicate. Expression ratios between the time points were determined with the REST tool (Pfaffl *et al.*, 2002).

Results

Occurrence of urease genes and urease activity

The occurrence of urease genes in *B. cereus* isolates was tested by PCR, using primers designed based on the *ureABC* genes of *B. cereus* ATCC 10987 (UreaseForw and UreaseRev, listed in Table 1). If the *ureABC* genes are present, a 958-bp DNA fragment will be amplified in the PCR. This fragment was indeed found in 10 out of 49 PCRs (-20%), and the corresponding strains were thus considered to harbour the *ureABC* cluster in their genomes (Table 2). The 10 *ureABC* PCR-positive strains and the *ureABC*-negative strains were subsequently tested for urease activity. In this test, the hydrolysis of urea and the concomitant production of ammonia resulted in an increase of the pH, which turned the pH indicator from yellow (low pH) to purple (high pH). This colour change was observed in 9 of the 49 tested strains, with the *ureABC*-negative strains and *ureABC*-positive strain P21S scoring negative after 24 h of incubation (Table 2). ATCC 10987 showed the highest urease activity, scoring positive within 6 hours of incubation. Notably, in all cases, the pH increase was inhibited by the addition of the urease inhibitor flurofamide, which indicated that alkalinisation was indeed dependent on urease activity.

Table 2. *B. cereus* strains tested for presence of *ureABC* genes; growth in minimal medium with glutamate, ammonium, and urea as sole nitrogen sources; and urease activity

B. cereus strain	Origin (Reference) [5]	*ureABC* PCR[1]	Growth in minimal medium with[1] Glutamate	Ammonium	Urea	Urease activity[1]
B434	Pasteurized milk	-	+	+	-	-
B436	Raw milk	-	+	+	-	-
B437	Pasteurized milk	-	+	+	-	-
B439	Pasteurized milk	-	+	+	-	-
B443	Pasteurized milk	-	+	+	-	-
ATCC 14579	Air[5a]	-	+	+	-	-
PAL2	Human feces[4]	-	+	-	-	-
PAL3	Human feces[4]	+	+	+	+	+
PAL5[2]	Patient feces[4]	+	+	+	+	+
PAL7	Human feces[4]	-	+	+	-	-
PAL17	Human feces[4]	-	-	+	-	-
PAL18[2]	Patient feces[4]	-	+	+	-	-
PAL20	Chilled food[5b]	+	+	+	+	+
PAL22	Chilled food[5b]	-	+	+	-	-
PAL25[3]	Human vomit[5c]	-	+	+	-	-
PAL26[2]	Meat loaf[6d]	-	+	+	-	-
PAL27[2]	Pea Soup[5e]	+	+	+	+	+
PAL28[2]	Food poisoning[5f]	-	+	+	-	-
17	Cream[5g]	-	-	+	-	-
55	Cream[5g]	+	+	+	+	+
59	Cream[5g]	+	+	+	+	+
61	Cream[5g]	+	+	+	+	+
72	Semi-skimmed Milk[5g]	-	+	+	-	-
132	Milk[5g]	-	+	+	-	-
43-92	Milk[5g]	+	+	-	-	+
401-92	Scrambled eggs[5g]	-	+	+	-	-
67-498	Scrambled eggs[5g]	-	+	-	-	-
1230-88	Stew[5g]	-	+	+	-	-
F450183	Clinical (PHLS) [5g]	-	+	+	-	-
DSM 11821T	Pasteurized Milk[5h]	-	-	+	-	-
KW85	Unknown	-	+	+	-	-
F5581	Unknown	-	+	+	-	-
TZ415	Chilled food[5b]	-	+	+	-	-
TZ426	Unknown	-	+	-	-	-
TZ427	Unknown	-	+	+	-	-
TZ428	Unknown	-	+	-	-	-
Z4234	Unknown	-	+	+	-	-
P21S	Unknown	+	+	+	+	-
Z421	Unknown	-	+	+	-	-
F3752A/86[3]	Emetic outbreak[5i]	-	+	+	-	-
B6/Ac	Unknown	-	+	+	-	-
F2797/87	Unknown	-	+	-	-	-
F3351/87[3]	Emetic outbreak[5i]	-	+	+	-	-
F3748/75	Human feces[5j]	-	+	+	-	-
F4635A/90	Unknown	-	+	-	-	-
F4628/90	Paella	-	+	-	-	-
F4626/90	Milk[5k]	-	+	+	-	-
F4623/90	Unknown	-	+	-	-	-
ATCC 10987	Spoiled cheese[5l]	+	+	-	-	+

[1] +, positive result, PCR product with correct size, growth observed (final OD >0.05), urease activity observed with cells grown in BHI; -, negative result, no PCR product, no growth observed (final OD <0.05), no urease activity observed with cells grown in BHI. The total numbers of positive strains were as follows: *ureABC* PCR, 10; glutamate, 46; ammonium, 39; urea, 8; and urease activity, 9.
[2] Diarrhoea associated strain
[3] Emetic strain
[4] Strain obtained from a RIVM project on the incidence and pathogenesis of gastroenteritis
[5] A (Frankland and Frankland, 1887); B (Choma *et al.*, 2000); C (Ash and Collins, 1992); D (Midura *et al.*, 1970); E (Spira and Goepfert, 1975); F (Granum *et al.*, 1993); G (Stenfors and Granum, 2001); H (Lechner *et al.*, 1998); I (Ehling-Schulz *et al.*, 2005); J (Finlay *et al.*, 2002); K (Bennik *et al.*, 1995); L (Rasko *et al.*, 2004)

Growth on urea as a nitrogen source

The utilization of urea, as well as that of glutamate and ammonium, as sole nitrogen sources was tested in minimal medium for the 49 *B. cereus* isolates (Table 2). All 49 strains were able to grow on glutamate and/or ammonium chloride as a nitrogen source with maltose as the sole carbon source. As expected, none of the *ureABC*-negative strains showed growth on urea as a nitrogen source. Of the 10 urease-positive isolates, 8 showed growth on urea and ammonium as sole nitrogen sources, whereas 2 isolates did not, including the sequenced *B. cereus* strain ATCC 10987. One urease PCR-positive strain (P21S) that did not display urease activity (see above) was able to utilize urea for growth. This was likely due to a low urease activity that was sufficient for growth but insufficient to elevate the pH in the urease activity assay. In summary, out of the 10 *ureABC*-positive strains identified in the collection of 49 environmental, food, and clinical *B. cereus* isolates, 8 were shown to utilize urea for growth.

Urease activity under acidic conditions

To elucidate a possible role for urease in the acid resistance of *B. cereus*, the *ureABC*-positive strains were subjected to two types of acid stress, i.e., growth at low pH and acid shock. The 10 urease-positive strains and the urease-negative type strain (ATCC 14579) were grown in BHI at pH 7 and pH 5 without and with extra urea (10 mM urea added) and with additional urea and flurofamide. Strains ATCC 14579 and ATCC 10987 were additionally tested in NB and NB with 10 mM urea added. Assessment of urea concentrations revealed BHI and NB to contain 1.1 mM and 0.5 mM urea, respectively. Neither the urease-positive strain ATCC 10987 nor the urease-negative strain ATCC 14579 showed differences in growth performance when the cells were cultured in BHI without additional urea, with extra urea, and with extra urea and flurofamide (Fig. 1). The other ureolytic strains showed similar growth profiles, and no urea-induced differences were detected (data not shown). Moreover, all growth performances appeared to be the same in the presence of flurofamide, indicating that urease activity did not contribute to the results obtained.

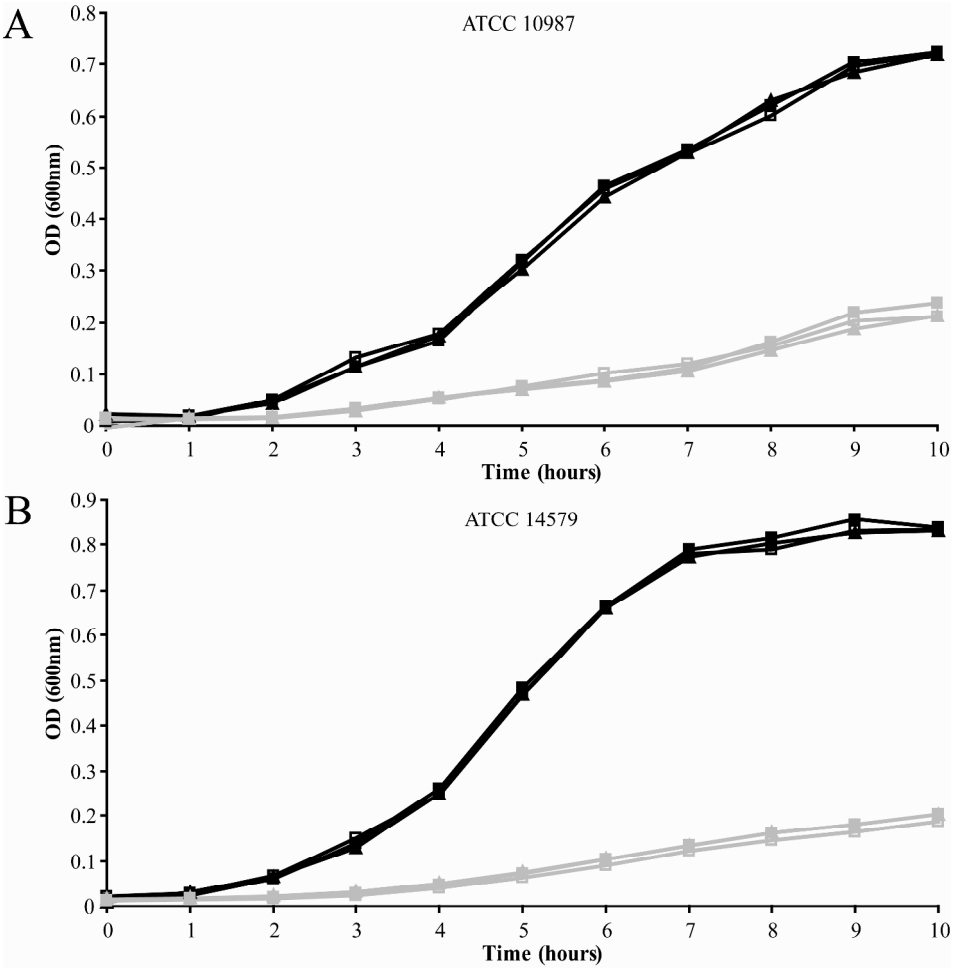

Fig. 1. Impacts of urea and urease inhibitor on the growth (OD600) of *B. cereus* strains ATCC 10987 (A) and ATCC 14579 (B) in BHI at pH 7 (black lines) and pH 5 (gray lines). Cultures without supplements are depicted by open squares; the closed squares represent cultures with the addition of urea, and the closed triangles represent cultures supplemented with urea and flurofamide.

The role of urease activity in acid shock survival was also examined. For this, the cells were grown overnight in BHI, and with the addition of hydrogen chloride, the pH was set at different acidic pH values, ranging from 2.5 to 4.8. Data obtained with stationary-phase cells of strain ATCC 10987 exposed to pH 4.5 are shown in Fig. 2. In the 30 min of exposure at pH 4.5, without and with extra urea present and with extra urea and flurofamide present, a similar decrease in the number of viable cells was observed. With mid-exponential-phase cells of ATCC 10987 exposed to pH 4.8, similar results were obtained,

i.e., urease activity did not contribute to survival capacity under the conditions tested (data not shown). Notably, similar observations were made for the nine other *ureABC*-positive strains (data not shown). These experiments revealed that urease activity, independent of the strains' capacities to metabolize the ammonia generated, did not contribute to *B. cereus* survival capacity under acid shock conditions.

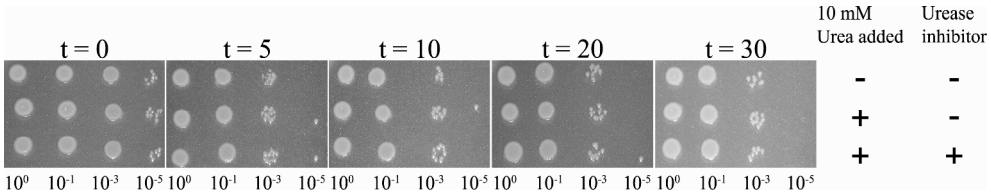

Fig. 2. Impacts of urea and urease inhibitor on survival of BHI-grown stationary-phase cells of *B. cereus* ATCC 10987 exposed to pH 4.5. Three exposed cultures, without added urea, with 10 mM added urea, and with added urea and flurofamide, as indicated at the right, were diluted and droplet plated. The time of low-pH exposure is depicted at the top of each plate, and the dilution factors are indicated below the plates. This experiment was performed in triplicate, and all replicates showed similar results; a typical result is shown here.

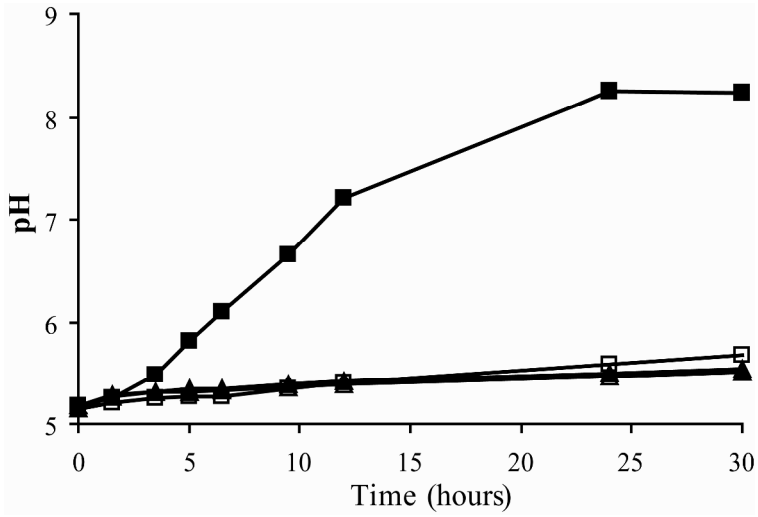

Fig. 3. Urease activity assay of *B. cereus* ATCC 10987 and ATCC 14579 in MES buffer. The ATCC 10987 cells in MES buffer supplemented with urea or with urea and flurofamide are depicted by closed squares and open squares, respectively. Closed triangles and open triangles represent the ATCC 14579 cells in MES buffer with urea or with urea and urease inhibitor, respectively.

Urease activity and expression of urease genes

The urease activities of *B. cereus* ATCC 10987 and ATCC 14579 (negative control) were assessed by measuring the pH increase of the medium due to the formation of ammonium. Cells from overnight cultures were resuspended in MES buffer supplemented with urea without and with the urease inhibitor flurofamide present. The pH was monitored for 30 h, during which the pH increased from 5.2 to 8.2 in the presence of ATCC 10987 cells, whereas with flurofamide present, such a pH increase was not observed (Fig. 3). This indicates that addition of 10 μM (final concentration) flurofamide is sufficient to inhibit the urease activity, and similar results were obtained with all other ureolytic *B. cereus* strains tested (data not shown). Based on these results, the urease enzyme activity of strain ATCC 10987 was determined at approximately 1 nmol min^{-1} mg $protein^{-1}$, indicating a very low activity level. The type strain ATCC 14579, which lacks the urease genes, did not provoke a pH increase under the conditions tested (Fig. 3).

At the indicated time points in the urease activity assay (Fig. 4A), expression of urease genes (Fig. 4B) was quantified by RT-RT-PCR (Fig. 4C and D). Total-RNA samples were taken from ATCC 10987 cells resuspended in MES buffer with 10 mM urea at pH 5.2 and MOPS buffer with 10 mM urea at pH 6.5. The general expression of the four tested urease cluster genes was very low compared to the expression of the reference genes *tufA* and *rpoA*. The RNA levels of *ureA*, *ureG*, *ureI*, and *nikT* were approximately 32 times lower than the RNA levels of *tufA* and *rpoA* (the average threshold cycles of time zero samples were as follows: *rpoA*, 16.71; *tufA*, 15.34; *ureA*, 22.54; *ureG*, 20.53; *ureI*, 19.64; and *nikT*, 20.47). Significant changes in expression, according to the REST tool, could be obtained despite the low mRNA levels of the urease genes. However, no increase of relative expression could be observed when cells were exposed to a lower pH. Figure 4C and D indicates that the urease cluster genes were down-regulated compared to *rpoA* and *tufA*, even though the cells displayed ureolytic activity. In addition, the relative expression ratios of the urease genes were also determined at different phases of growth under aerobic conditions, and samples were taken from BHI cultures upon reaching ODs of 0.2, 0.5, and 5 and after overnight incubation. Under anaerobic growth conditions, samples were taken from BHI cultures upon reaching ODs of 0.2, 0.5, and 1 and after overnight incubation. Again, low levels of mRNAs of the urease genes were observed with no significant changes in the relative expression ratios of the urease genes during different growth stages in BHI under the conditions tested (data not shown). In conclusion, under a wide range of conditions tested, the expression of urease cluster genes was low, and no urea-, pH-, oxygen-, or nitrogen-dependent induction of expression could be observed.

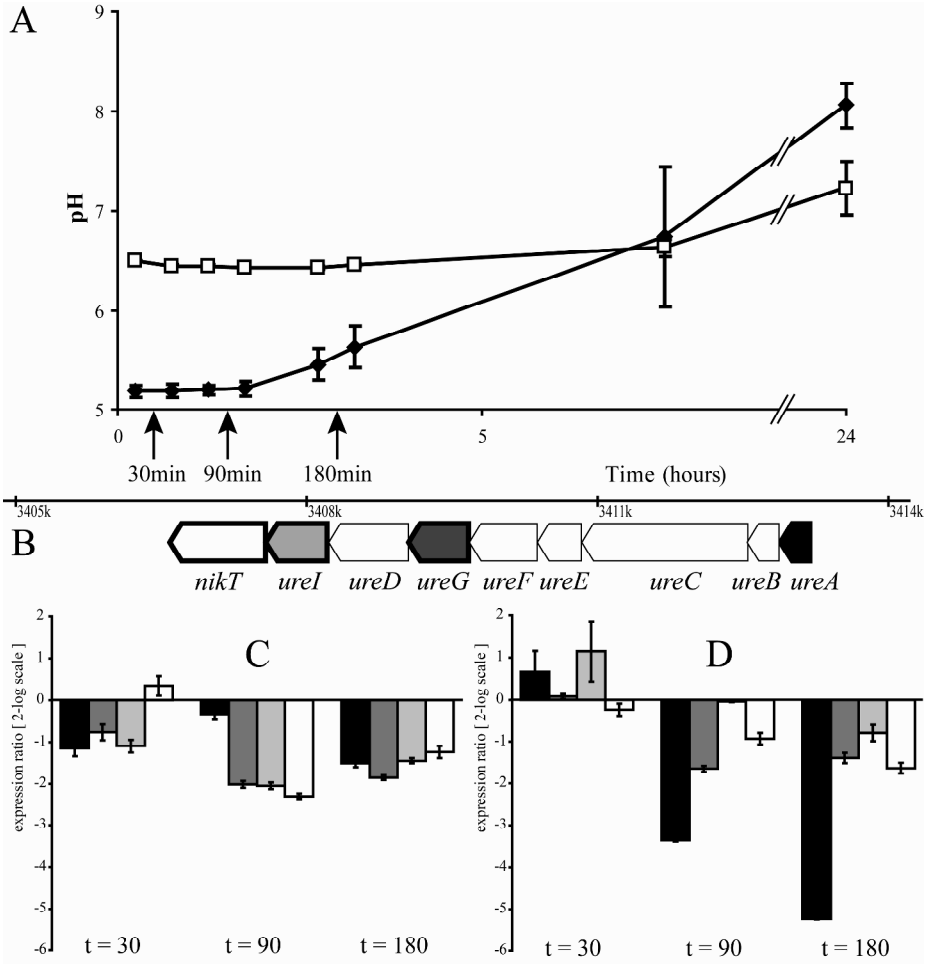

Fig. 4. Impacts of pH and urease activity on the relative expression ratios of urease cluster genes of *B. cereus* ATCC 10987. (A) pH increase over time of ATCC 10987 cells in MES buffer with 10 mM urea at pH 5.2 (closed diamonds) and of cells in MOPS buffer with 10 mM urea at pH 6.5 (open squares). The error bars represent the standard deviations between duplicate experiments, and the time points at which RNA samples were taken are indicated by arrows. (B) Positions of the genes investigated with RT-RT-PCR within the urease cluster: *ureA* (black), *ureG* (dark gray), *ureI* (light gray), and *nikT* (white). The other genes (*ureB*, *ureC*, *ureE*, *ureF*, and *ureD*) are indicated in white, and the gene aliases are indicated at the bottom. (C and D) Relative expression ratios compared to time zero of *ureA*, *ureG*, *ureI*, and *nikT* (the colours correspond to those in panel B) of ATCC 10987 cells exposed to pH 6.5 in MOPS buffer with 10 mM urea (C) and to pH 5.2 in MES buffer with 10 mM urea (D) at 30, 90, and 180 min (left, middle, and right, respectively). The expression ratios are expressed in $\log_2$ scale, and the error bars represent the standard errors between the duplicate experiments.

Discussion

This study shows that 10 of the 49 (~20%) tested clinical, food, and environmental *B. cereus* isolates harbour *ureABC* genes. Notably, only eight of these were able to use urea as a sole nitrogen source, whereas two of the strains, including the sequenced *B. cereus* strain ATCC 10987, could not. Remarkably, these two strains displayed ureolytic activity, and their failure to use urea as a nitrogen source can be explained by the inability to utilize ammonium for growth. The ammonium-negative phenotype of ATCC 10987 is possibly due to the lack of aspartate ammonia-lyase (EC 4.3.1.1) and the inability to utilize glutamine as a sole nitrogen source (Mols *et al.*, 2007). The urease-positive strains that are capable of utilizing ammonium for growth conceivably incorporate ammonium into nitrogen metabolism via glutamine or aspartate (Fig. 5). Among the 49 isolates tested, eight strains have been associated with outbreaks (five diarrheal and three emetic strains), and only strains PAL5 and PAL27 (both diarrheal) were shown to be ureolytic. However, the limited number of disease-associated (diarrheal) *B. cereus* strains used in our study does not allow conclusions to be drawn as to the correlation (if any) between the presence of urease genes (and ureolytic activity) and the capability to cause disease.

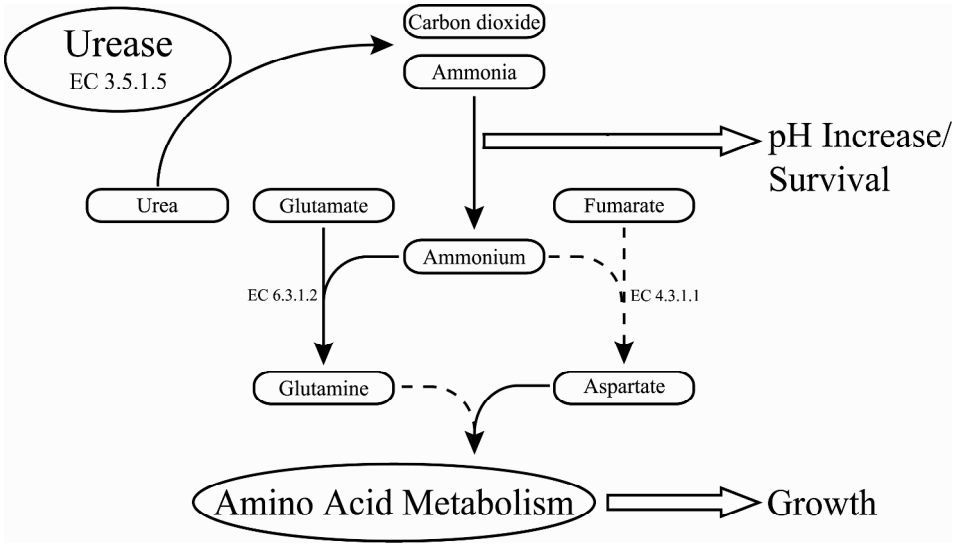

Fig. 5. Schematic representation of the functions of urease in *B. cereus*. Urea is hydrolyzed by urease (EC 3.5.1.5), forming carbon dioxide and ammonia. Subsequently, ammonia is converted into ammonium, which causes the pH to increase and may serve low-pH survival. Ammonium can be used as a nitrogen source and may be included in nitrogen metabolism via two possible routes supporting bacterial growth. The involved enzymes, glutamine synthetase and aspartate-ammonia lyase, are indicated by enzyme numbers (6.3.1.2 and 4.3.1.1, respectively). Note that the sequenced ATCC 10987 strain cannot use ammonium as a sole nitrogen source. The routes and/or enzymes missing in ATCC 10987 are indicated by dashed lines.

The formation of ammonium out of ammonia results in an elevation of the pH (Fig. 5), which may increase the fitness of ureolytic strains in acidic environments. It has been proposed (Rasko *et al.*, 2004) that the acquisition of the urease genes may have an impact on the survival of *B. cereus* strain ATCC 10987 under acidic conditions, e.g., the human stomach, as observed for *H. pylori* (Tsuda *et al.*, 1994). However, growth of the ureolytic strains in urea-containing media with and without urease inhibitor at a low pH revealed no differences in fitness, i.e., none of the strains showed significant differences in the growth rate or final OD reached under the conditions tested. Furthermore, no differences in survival capacity were observed between cells of the ureolytic strains exposed to an acid shock in the presence of extra urea and cells exposed to an acid shock while urease activity was blocked by flurofamide. The results obtained can be explained by the low ureolytic activity displayed by the *B. cereus* strains tested. This is in contrast to the results obtained for *S. salivarius* (Chen *et al.*, 2000) and *H. pylori* (Tsuda *et al.*, 1994), where high ureolytic activity enabled the bacterial cells to cope with acid stress and, in the case of *H. pylori*, to colonize the human stomach. The expression of urease genes can be constitutive, regulated by the presence of urea, pH, and/or nitrogen availability (Collins and D'Orazio, 1993). The expression of the urease cluster in *B. cereus* ATCC 10987 appeared to be constitutive, because no up-regulation of the urease genes was observed after exposure to urea or acid downshift and under conditions of nitrogen limitation. Furthermore, the expression of the urease genes in *B. cereus* is generally very low compared to that of the housekeeping genes *rpoA* and *tufA*. The constitutive expression of the urease genes of *B. cereus* is in contrast with the regulation of the urease genes found in *Proteus mirabilis* (Collins and D'Orazio, 1993), *S. salivarius* (Sissons *et al.*, 1990), and *B. subtilis* (Collins and D'Orazio, 1993). The urease genes of *P. mirabilis* are expressed only when urea is present in the growth medium. The UreR regulator as present in the genome of *P. mirabilis* is involved in the urea-dependent induction of the urease cluster (Collins and D'Orazio, 1993). Such a UreR regulator is absent in the genome of *B. cereus* ATCC 10987, as previously noted by Rasko and colleagues (2004). In *S. salivarius* and other bacteria, urease expression is regulated by the pH; for example, at pH 5.5, urease activity increased 100-fold compared to that at pH 7.0 in *S. salivarius* (Sissons *et al.*, 1990). In *B. subtilis*, the expression of the urease operon is induced by nitrogen limitation (Atkinson and Fisher, 1991) and regulated by a key transition phase transcription regulator, Spo0H (sigma factor σ^H) (Wray *et al.*, 1997). This results in elevated expression of the urease genes in *B. subtilis* during stationary phase, which has also been shown for *Y. enterocolitica* (de Koning-Ward and Robins-Browne, 1997). Screening of the genome of ATCC 10987 with a *B. cereus*-specific σ^H promoter consensus revealed that this strain does not harbour a σ^H promoter in front of the urease cluster (M. Tempelaars and T. Abee, unpublished results), and this corresponds to the absence of up-regulation of the urease genes in transition and stationary phase in *B. cereus*. Even though a dual role of ureolytic activity in nitrogen metabolism and in acid stress survival has been described for *Y. enterocolitica* (Young *et al.*, 1996), *S. salivarius* (Chen *et al.*, 2000), and *H. pylori* (Williams *et al.*, 1996), we can conclude that none of the ureolytic

B. cereus strains displayed increased fitness under acidic conditions or showed increased acid shock survival in the presence of urea. This is most likely linked to the low level of expression of the urease genes, the lack of modulation of their expression, and the resultant low level of ureolytic activity. Therefore, we conclude that the main role of *B. cereus* urease is in nitrogen metabolism, so that ammonia may be provided to the cells in nitrogen-limited environments.

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Chapter 4

Comparative analysis of transcriptional and physiological responses of *Bacillus cereus* to organic and inorganic acid shocks

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Submitted for publication

Abstract
Comparative phenotype and transcriptome analyses were performed with *Bacillus cereus* ATCC 14579 exposed to pH 5.5 set with different acidulants including hydrochloric acid (HCl), lactic acid (HL) and acetic acid (HAc). Phenotypes observed included a decreased growth rate (with HCl), bacteriostatic and bactericidal conditions, with 2 mM undissociated HAc or HL, and 15 mM undissociated HAc, respectively. In the latter condition a concomitant decrease in intracellular ATP levels was observed. The transcriptome analyses revealed general and specific responses to the acidulants used. The general acid stress response includes modulation of pyruvate metabolism with activation of the butanediol fermentation pathway, and an oxidative stress response that was, however, more extensive in the bacteriostatic and bactericidal conditions. HL-specific and HAc-specific responses include modulation of metabolic pathways for amino acid metabolism. Activation of lactate, formate, and ethanol fermentation pathways, alternative electron-transport chain components and fatty acid biosynthesis genes was noted in the presence of 15 mM undissociated HAc. In conclusion, our study has provided insights in phenotype-associated, and general and acidulant-specific responses in *B. cereus*.

Introduction

Bacillus cereus is a spore-forming Gram-positive bacterium that can cause diarrheic or emetic symptoms of food poisoning (Kotiranta *et al.*, 2000). The emetic syndrome is caused by consumption of cereulide-contaminated food, while the diarrhoeal syndrome is caused by enterotoxins that are produced by *B. cereus* cells in the small intestine (Stenfors Arnesen *et al.*, 2008). Food product groups that form a potential risk for *B. cereus* infections include ready-to-eat foods containing rice or pasta, milk and milk products, flavourings, pastry, vegetables and vegetable products (Wijnands *et al.*, 2006). *B. cereus* must be able to withstand low pH conditions such as encountered in foods acidified during food processing and conservation. Furthermore, enterotoxic *B. cereus* strains have to survive gastric transit to reach the human intestine. Therefore, a thorough understanding of the *B. cereus* response to acid shock may aid in defining safe food preservation conditions.

Organic acids like lactic acid (HL) and acetic acid (HAc) are often used as food preservatives. The pK_a of acids (3.86 for HL and 4.76 for HAc) determines the ratio between dissociated and undissociated forms at a given pH. The undissociated form can diffuse into the cell where it dissociates, releasing protons, until an equilibrium is reached. Different theories regarding the antimicrobial activity of organic acids have been raised, such as dissipation of the proton motive force, including lowering of the intracellular pH (pH_i), and intracellular accumulation of anions resulting in end-product inhibition (Brul and Coote, 1999; Cotter and Hill, 2003).

The responses of Gram-positive bacteria to acid stress are diverse (Cotter and Hill, 2003) and may include activation of proton pumps and protein repair systems, modification of cell membrane composition, production of alkali, and alteration of metabolism. However, the acid stress response of *B. cereus* ATCC 14579 has not been studied extensively. Available information is limited to the acid tolerance response, which includes modulation of pH_i and protein synthesis (Browne and Dowds, 2002; Thomassin *et al.*, 2006). Moreover, most information on the bacterial acid stress response is related to acid shock treatment or acid tolerance resulting in inhibited growth, while there is no information available on the bacteriostatic and/or bactericidal response to acid stress, although this is most relevant for food preservation.

In this study, comparative phenotype and transcriptome analyses were performed with *Bacillus cereus* ATCC 14579 exposed to pH 5.5 set with different acidulants including hydrochloric acid (HCl), lactic acid (HL) and acetic acid (HAc). Phenotypic responses included decreased growth rates, bacteriostatic and bactericidal conditions, and these were linked with transcriptome analyses, providing insights in phenotype-associated, and general and acidulant-specific responses in *B. cereus*.

Materials and methods

Bacterial strains and growth conditions
Bacillus cereus type strain ATCC 14579 was obtained from the American Type Culture Collection and grown at 30ºC, 200 rpm in Brain Heart Infusion (BHI, Becton Dickinson, France) broth, buffered at pH 7.1 with 100 mM sodium phosphate. Cells were grown to exponential phase, at which the culture was acidified to pH 5.5. pH 5.5 acid shock was achieved by addition of 0.238% (v/v) 12 M HCl (Merck, Germany), by addition of 0.698% (v/v) HL (PURAC FCC 80; PURAC, The Netherlands), which resulted in 2 mM undissociated HL, by addition of 0.205% (v/v) 12 M HCl in combination with 0.074% (v/v) HAc (Merck, Germany), which resulted in 2 mM undissociated HAc, or by addition of 0.571% (v/v) HAc, which resulted in 15 mM undissociated HAc. Impact of acid exposure on growth of *B. cereus* was assessed by measuring the optical density at 600 nm (OD, Novaspec II, Pharmacia Biotech, Germany) of the cultures at different time intervals. The survival upon acid shock was investigated by plating samples, taken at different time intervals, on BHI agar plates (15 g l^{-1} bacteriological agar, Oxoid, England) and overnight incubation at 30°C.

ATP measurements
The ATP concentration of samples obtained from the acid shocked cultures at different time intervals was measured. ATP measurements were performed using a Biocounter M2500 (Lumac BV, The Netherlands) in combination with the Microbial biomass kit (Celsis, The Netherlands) according to instructions of the manufacturer. The conversion of ATP to AMP by luciferase was measured in Relative Light Units (RLU). Using a range of ATP standards (100 nM – 100 μM), the unknown ATP concentration of the sample was determined. An integration and measuring period of 10 seconds was applied. Total ATP was measured by adding 2 ml absolute ethanol (Merck, Germany) to 1 ml of culture. After an incubation period of 10 minutes at -20ºC, 20 μl of the ethanol culture mixture was added to 180 μl of water and ATP was measured. ATP background levels were obtained by determining the ATP concentration of the supernatant.

RNA isolation, cDNA labelling and microarray hybridization
Samples for RNA isolation were taken at OD 0.5 just before addition of the acidulants and at 10, 30, and 60 min of exposure. Twenty ml of the culture was used for RNA isolation. After pelleting the cells in 30 sec (Eppendorf centrifuge 5804 R, Eppendorf, Germany), the supernatant was discarded and the cell pellets were resuspended in 1 ml Tri-reagent (Ambion, UK). The resuspended pellets were quick frozen in liquid nitrogen and stored at -80ºC until RNA isolation. RNA isolation, cDNA labelling and microarray hybridization were performed as described previously (van Schaik *et al.*, 2007). Custom-made Agilent *B. cereus* microarrays (GEO accession number GPL7679) were hybridized and after washing

scanned in an Agilent microarray scanner (G2565BA). Data were extracted using Feature Extraction Software Version 8, which includes LOWESS normalization of the raw data.

Analysis of microarray data

After removal of the data for different controls printed on the microarray slides, the normalized data for each spot from the microarrays were analyzed for statistical significance using the Web-based VAMPIRE microarray suite (Hsiao *et al.*, 2005). A spot was found to be differentially expressed between two samples when the false discovery rate was smaller than 0.05. Subsequently, the data for the single spots were integrated to obtain expression ratios for an open reading frame. An open reading frame was found to be differentially expressed when all spots representing the open reading frame were significantly differentially expressed between the samples. The expression ratios of an open reading frame from duplicate experiments were averaged to obtain a single expression ratio per open reading frame. Finally, changes of 2-fold (for up-regulated genes in the stress condition) and 0.5-fold (for down-regulated genes in the stress condition) were also introduced as significance limits. GeneMaths XT (version 1.6.1, Applied Maths, Belgium) was used for visualization, clustering and further analysis of the microarray data. The dendrogram of the microarray sets was generated using average linkage hierarchical clustering and the Euclidian distance matrix. Hierarchical clustering (complete linkage, Euclidian distance) of all genes was used to identify groups of genes with similar transcription profiles. The overrepresentation of functional classes within the groups of genes with similar expression profiles was evaluated using FIVA (Blom *et al.*, 2007) (results shown in *Supplementary material*).

Results

Growth and viability

The effects of pH 5.5 acid shock using different acidulants on *B. cereus* growth were determined (Fig. 1). pH 5.5 was set with HL and HAc, but because of their different pK_a this resulted in 2 and 15 mm undissociated acid, respectively. In addition, a combination of HAc and HCl was used to acidify the cultures to pH 5.5 to obtain a condition with 2 mM undissociated HAc. Adding HCl as acidulant had the mildest effect and led to a decreased growth rate compared to the untreated control. Addition of HL (2 mM undissociated acid) or HAc (2 mM or 15 mM undissociated acid) resulted in growth arrest. However, prolonged incubation revealed a clear difference between the conditions. In the conditions with 2 mM undissociated acids growth was resumed whereas the condition with 15 mM undissociated HAc did not show an increase of OD after 24 hours (data not shown). Assessment of colony forming units (cfu) after 24 hours showed the unstressed control cultures to have $9.2 \cdot 10^8$ cfu ml^{-1}, HCl-shocked cultures to have $9.0 \cdot 10^8$ cfu ml^{-1}, the HL-shocked cultures $4.0 \cdot 10^8$ cfu ml^{-1}, the HAc/HCl-shocked cultures $2.6 \cdot 10^8$ cfu ml^{-1}, while the cfu of the 15 mM undissociated HAc shocked cultures were below the detection limit of

10^4 cfu ml^{-1} (data not shown). Assessment of the pH after 24 hours showed that the pH of the unstressed control cultures had risen to pH 8.8, that of the HCl-shocked cultures was pH 8.5, that of the HL-shocked cultures pH 7.2, the HAc/HCl-shocked cultures were pH 7.1 and that the pH of the HAc-shocked cultures was not increased and remained at pH 5.5 (data not shown).

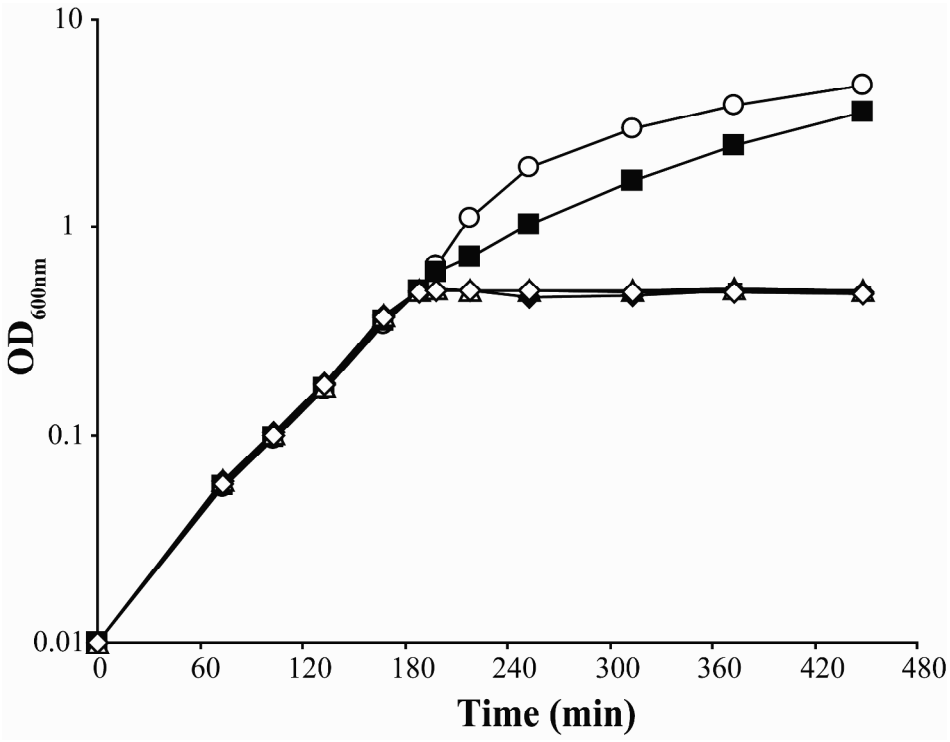


Fig. 1. Impact of acid shock on growth of *B. cereus*. Upon reaching OD 0.5, the pH of the cultures was adjusted to pH 5.5 using HCl (filled squares), HL (open triangles), HAc/HCl (filled diamonds) or HAc (open diamonds) as acidulants. The non-stressed control culture is depicted with open circles. The mean values of four cultures are shown with corresponding standard deviations indicated by error bars.

Effect of acid shock on ATP levels

The initial physiological responses of *B. cereus* upon exposure to the different conditions used were studied in more detail by determining the viability through plate counts and the cellular energy status through ATP measurements (Fig. 2). Samples were taken directly before and 10, 30, and 60 minutes after exposure. In the presence of 2 mM undissociated HL or HAc/HCl, viable counts remained constant in 60 minutes of exposure, confirming the bacteriostatic conditions observed in Fig 1. In contrast, in the presence of HCl viable

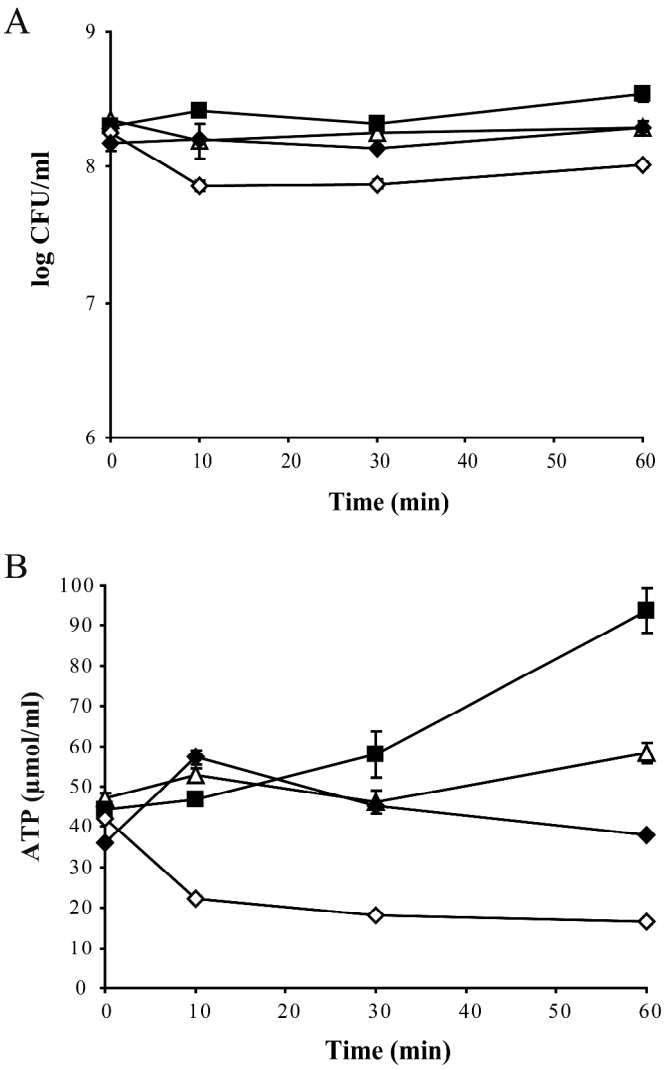


Fig. 2. Plate counts (A) and ATP measurements (B) upon acid shocks in time. Upon reaching OD 0.5, the pH of the cultures was adjusted to pH 5.5 using HCl (filled squares), HL (open triangles), HAc/HCl (filled diamonds) or HAc (open diamonds) as acidulants. Data are mean values from duplicate cultures and error bars indicate standard deviations.

counts were significantly higher (P<0.02), indicating growth. Finally, in the presence of 15 mM undissociated organic acid the cfu were significantly lower (P<0.006), indicating bactericidal conditions. To determine the effect of the different stress conditions on the energy status of the cells, ATP measurements were performed (Fig. 2B). The HCl-exposed

cultures that were continuing growth showed an increase of ATP levels after 30 min. The ATP levels in the HL and HAc/HCl exposed cultures remained constant and exposure to 15 mM undissociated HAc resulted in a significant decrease in ATP levels ($P<0.005$). Based on these results we conclude that only a pH 5.5 acid shock in the presence of 15 mM undissociated HAc caused depletion of ATP with concomitant loss of viability.

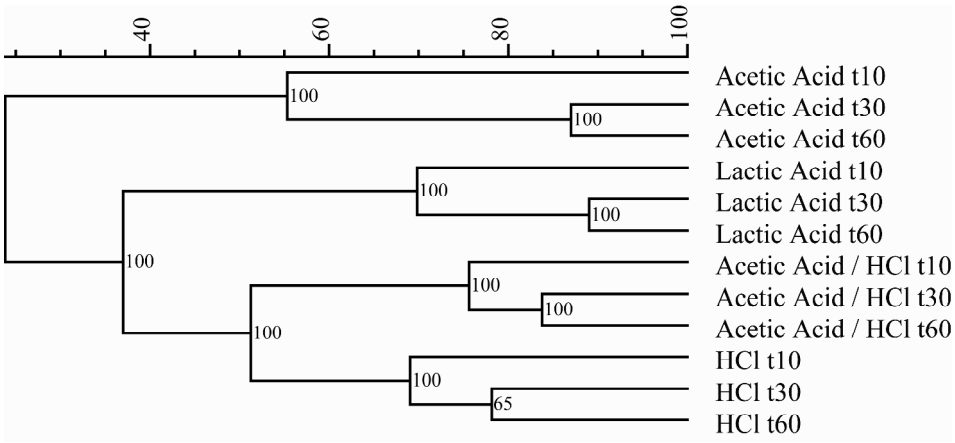


Fig. 3. Dendrogram showing clustering of up- and down-regulated genes for the different acid shock conditions at time points 10, 30, and 60 minutes relative to time 0 just before acid shock.

Microarray analysis

Effect of acid shock on gene expression

To study the impact of the different acid shock treatments on gene expression, samples taken after 10, 30, and 60 min exposure were compared to a reference sample taken immediately before acid exposure (GEO accession number GSE15140). The datasets of gene expression relative to the reference condition were used to construct a dendrogram (Fig. 3) showing differences in time and between treatments. The bactericidal HAc shock grouped apart from the other treatments that did not affect viability. From the three remaining conditions, the HAc/HCl shock and the HCl shock grouped closest together. This was unexpected, as it did not correlate with physiological responses for which the 2 mM undissociated organic acid treatments responded similar and differed from the HCl shock. This overlapping response of the HAc/HCl and HCl treatments may be due to the increase of Cl^- ions in both conditions due to the addition of HCl as the (co)acidulant. Hierarchical clustering of all genes expressed upon all acid shocks revealed groups of genes with similar expression profiles (Fig. 4) (results presented in detail below). Furthermore, it showed that the ratios obtained from samples exposed for 30 minutes represented the majority of processes that were affected at earlier and later stages, i.e., 10 and 60 minutes of exposure,

respectively. Therefore, ratios obtained from samples exposed for 30 minutes were chosen for a more detailed analysis of the effects on gene expression levels.

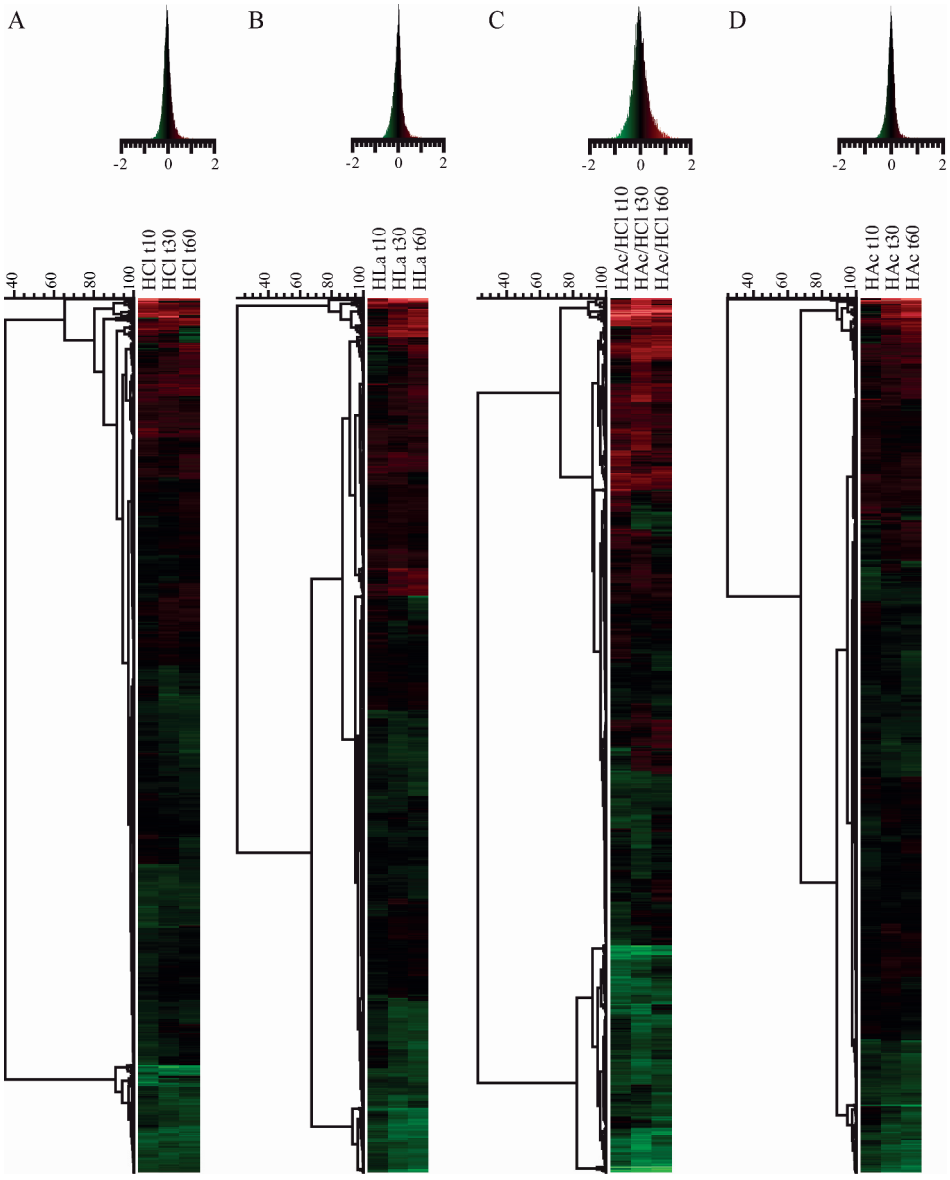


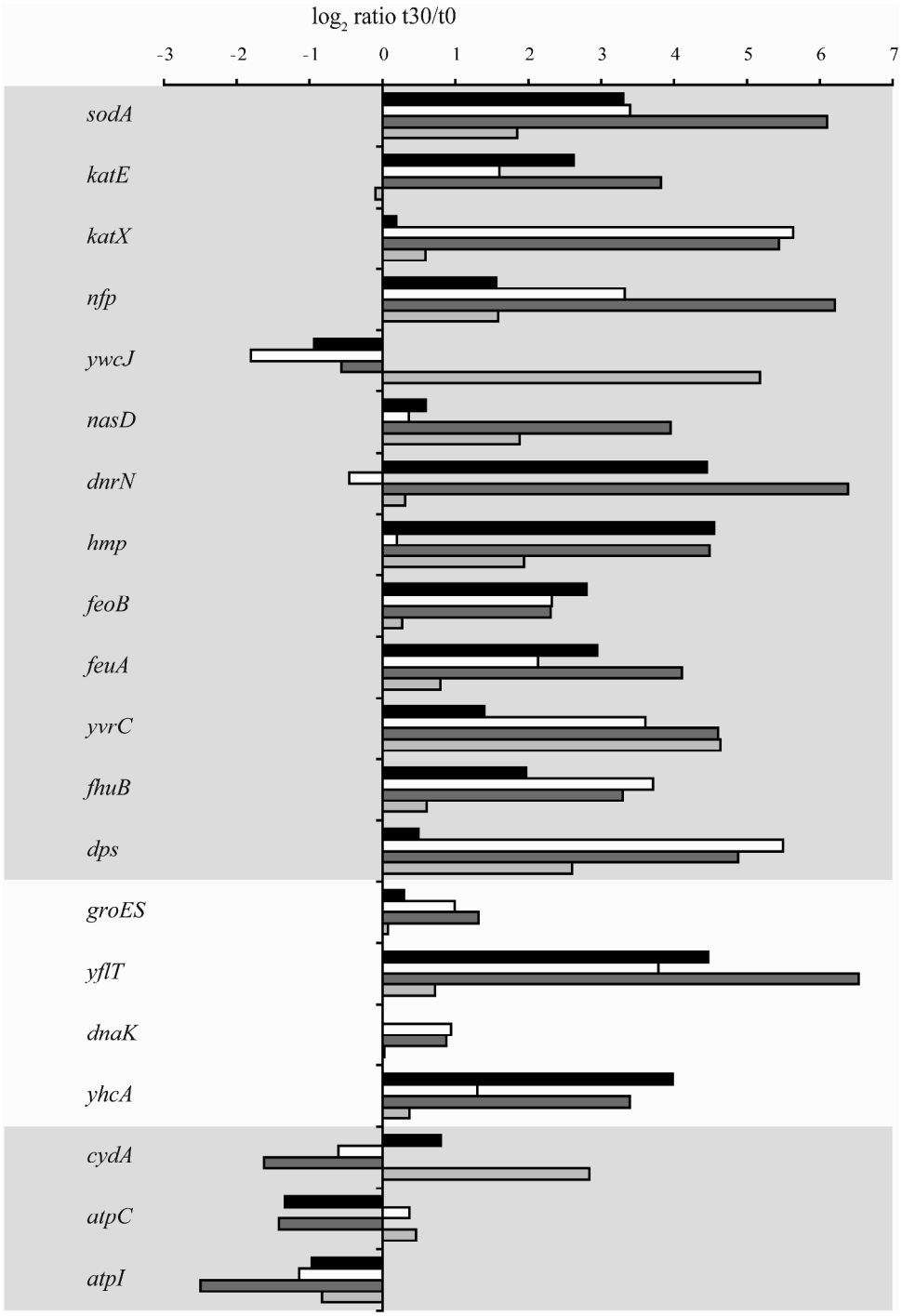
Fig. 4. Hierarchical clustering of all genes expressed upon HCl (A), HL (B), HAc/HCl (C) and HAc (D) treatments. Log_2 ratios of all genes at 10, 30 and 60 min of exposure were clustered and displayed colorimetrically for the different acidulants used. Histograms with colour scale are shown above.

Gene expression associated with acid shock: the general acid shock response

The effects of acid shock were determined by selecting all genes having significant differential expression in all acid exposures or in non-lethal exposures specifically (see *Supplementary material*). Genes differentially expressed in non-lethal conditions were determined by selecting the genes showing significant differential expression in HCl, HL, and HAc/HCl and not showing a similar significant differential expression in HAc. A set of 25 genes (17 up and 8 down) was differentially expressed in all acid shock conditions and a set of 146 genes (86 up and 60 down) was differentially expressed in all non-lethal acid shock conditions. The largest impact was shown on the expression of genes involved in energy metabolism, oxidative and general stress response (up-regulated) and nucleotide metabolism and cell-wall biogenesis (down-regulated) (Fig. 5). Energy metabolism was mainly affected in pyruvate metabolism and TCA cycle (Fig. 6). Genes involved in nucleotide transport and metabolism were down-regulated upon all acid shocks, which is in line with the observed inhibition of growth (Fig. 1). Cell envelope biogenesis was affected in teichoic acid and capsular polysaccharide synthesis genes. Notably, expression of the F_1F_0-ATPase was down-regulated in non-lethal acid shocks. Based on observations that acid stress conditions induced, next to a general stress response involving σ^B and ClpBC, an oxidative stress response involving superoxide dismutase, catalase and iron homeostasis proteins, we conclude that reactive oxygen species may be formed. At the same time the pyruvate metabolism is changing dramatically, including induction of the TCA cycle and concurrent induction of fermentation pathways. This may be required for maintaining intracellular ATP levels (Fig. 2B) and/or the redox balance.

Gene expression associated with bactericidal conditions

Genes associated with bactericidal conditions were defined as differentially expressed genes in cells exposed to 15 mM undissociated HAc having different expression upon exposure to 2 mM undissociated organic acids and HCl. 137 genes (60 up-regulated and 77 down-regulated) could be associated with the bactericidal condition (see *Supplementary material*). The largest impact was on expression of genes involved in energy metabolism and electron transport (up-regulated) and on genes of unknown function (down-regulated) (Fig. 5; Fig. 6). Different fermentation pathways were induced via up-regulation of genes encoding L-lactate dehydrogenases, lactate permease, and alcohol dehydrogenases. Expression of electron transport genes was also up-regulated upon lethal HAc stress. It is conceivable that the concerted activity of NAD(P)-dependent dehydrogenases and the electron transport system is used as ultimate response to pump protons out of the cell or to restore the redox balance. Other cellular processes that were specifically affected by lethal HAc stress were transport mechanisms and cell membrane biogenesis.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-2
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ywcJ
nasD
dnrN
hmp
feoB
feuA
yvrC
fhuB
dps
groES
yflT
dnaK
yhcA
cydA
atpC
atpI

Fig. 5. The response of oxidative stress, general stress response and electron transport associated genes to the acid shock conditions. Bars indicate $\log_2$ t30/t0 ratio of cultures acidified with HCl (black bars), 2 mM undissociated HL (white bars), 2 mM undissociated HAc (HAc/HCl, dark grey bars), and 15 mM undissociated HAc (light grey bars). Oxidative stress associated genes are represented by genes encoding: superoxide dismutase (*sodA*, BC5445), catalase E (*katE*, BC0863), catalase X (*katX*, BC1155), nitroreductase family protein (*nfp*, BC1952), nitrite transporter (*ywcJ*, BC1308), nitrite reductase (*nasD*, BC1251;), nitric oxide-dependent regulator (*dnrN*, BC2137), nitric oxide dioxygenase (*hmp*, BC1448), ferrous iron transport gene (*feoB*, BC0707), iron dicitrate transporter (*feuA*, BC3738), ferrichrome-transport protein (*yvrC*, BC4363), ferrichrome transport gene (*fhuB*, BC4362), and dps-like protein (*dps*, BC5044). Genes associated with other stresses are represented by genes encoding: chaperone protein GroES (*groES*, BC0294), general stress protein 17M (*yflT*, BC0998), chaperone protein DnaK (*dnaK*, BC4312), and multidrug resistance protein (*yhcA*, BC4568). Electron transport related genes are depicted by genes encoding: cytochrome D ubiquinol oxidase (*cydA*, BC1938), ATP synthase genes *atpC* (BC5305) and *atpI* (BC5313).

Gene expression associated with bacteriostatic conditions

Genes associated with bacteriostatic conditions, i.e., the presence of 2 mM undissociated organic acids, were defined as all similarly differentially expressed genes (all at least two-fold up or all at least two-fold down) of the HL and HAc/HCl exposed cells having different expression from the HCl and HAc exposed cells. Our data set had 224 genes (82 up-regulated & 142 down-regulated) that were differentially expressed in the 2 mM undissociated organic acid exposures (see *Supplementary material*). The largest impact was on expression of genes involved in oxidative stress and redox balancing (up-regulated), and amino acid transport and metabolism and membrane and cell envelope biogenesis (down-regulated). In summary, gene expression associated with bacteriostatic organic acid stress conditions differs from growth-inhibiting inorganic acid stress conditions and lethal HAc exposure by an extended oxidative stress response, which includes the expression of an additional catalase, which is conceivably required to counteract increased oxidative damage. This response, together with changes in amino acid and oligopeptide uptake and metabolism and down-regulation of lipid metabolism seems to allow for survival and maintenance of the energy status, while cell growth was inhibited.

Gene expression specific for HL exposure

Genes associated with HL response were defined as all differentially expressed genes of HL exposure having different expression in the HAc/HCl, HAc, and HCl exposure. Our data set had 196 genes (55 up-regulated and 141 down-regulated) that were differentially expressed upon HL exposure (see *Supplementary material*). The largest impact was on expression of genes involved in amino acid metabolism, most notably the arginine pathway, transport mechanisms and genes of unknown function (up-regulated). Several genes involved in glycolysis were also moderately up-regulated. In cell envelope biogenesis there was an up-regulation of the murein hydrolase exporter and regulator genes, but several other genes involved in cell envelope biogenesis were down-regulated. Other differentially expressed

genes were mainly putative transcription regulators, ABC transporters with unknown substrate or with an unknown function.

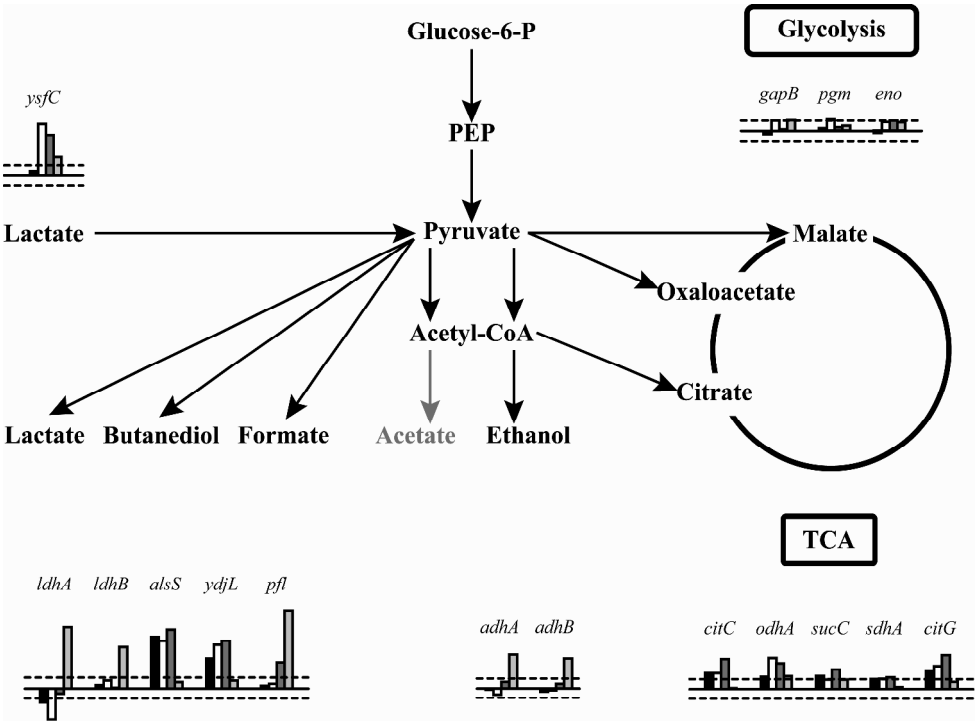

Fig. 6. The responses of pyruvate metabolism genes to the acid shock conditions. Bars indicate $\log_2$ t30/t0 ratio of cultures acidified with HCl (black bars), 2 mM undissociated HL (white bars), 2 mM undissociated HAc (HAc/HCl, dark grey bars), and 15 mM undissociated HAc (light grey bars). Dotted lines indicate 2-fold ($\log_2 = 1$) induction (above) or repression (below). Glycolysis genes are represented by genes encoding: glyceraldehyde 3-phosphate dehydrogenase (*gapB*, BC4583), phosphoglycerate mutase (*pgm*, BC5136), and enolase (*eno*, BC5135). TCA cycle genes are depicted by: isocitrate dehydrogenase (*citC*, BC4593), 2-ketoglutarate dehydrogenase (*odhA*, BC1252), succinyl-CoA synthetase (*sucC*, BC3834), succinate dehydrogenase (*sdhA*, BC4517), and fumarate hydratase (*citG*, BC1712). Pyruvate can be converted to lactate, butanediol formate, acetate and ethanol. The genes facilitating these conversions are represented by: L-lactate dehydrogenases *ldhA* (BC4870) and *ldhB* (BC4996) for lactate formation, acetolactate synthase (*alsS*, BC0883) and butanediol dehydrogenase (*ydjL*, BC0668) for butanediol formation, formate acetyltransferase (*pfl*, BC0491) for formate formation, and alcohol dehydrogenases *adhA* (BC2220) and *adhB* (BC4365) for ethanol formation. Genes encoding acetate forming enzymes were not differentially expressed and therefore acetate is shown in grey. Lactate conversion forming pyruvate is depicted by 2-hydroxy-acid oxidase encoding *ysfC* (BC1297).

Gene expression associated with non-lethal HAc exposure

Genes associated with 2 mM undissociated HAc response were defined as all differentially expressed genes of the HAc/HCl dataset having different expression in the HCl data set. This data set had 1430 genes (696 up-regulated and 734 down-regulated) that were differentially expressed upon HAc/HCl exposure (see *Supplementary materials*) compared to exposure to the same pH acidified with only HCl, indicating a tremendous impact of HAc on gene expression. The largest impact was on expression of genes involved in oligopeptide and amino acid transport and metabolism, but other pathways, most notably in redox balancing and lipid and energy metabolism, were also affected. The exposure to HAc/HCl also induced the expression of GroES and GroEL genes. There was a clear shift in carbohydrate transport and metabolism with genes encoding sugar transporters for fructose, glucose, lichenan, and trehalose being down-regulated while another glucose transport gene (BC5320) was up-regulated. In electron transport induction of expression of nitrate reductase and molybdopterin biosynthesis genes indicated a switch to anaerobic respiration under aerobic conditions and/or a rearrangement in nitrogen metabolism. For nucleotide transport and metabolism there were various changes in gene expression for purine, and pyrimidine metabolism (see *Supplementary material*). Translation, ribosomal structure and biogenesis was affected as shown by down-regulation of ribosomal protein gene expression, which is opposite from the response to HL exposure where these genes were moderately up-regulated. Also the expression of several ABC transporters with unknown substrate specificities was opposite from the response to HL exposure.

Discussion

In this study, *B. cereus* ATCC 14579 was exposed to pH 5.5 set with different acidulants. Depending on the acidulant used, there was a clear difference in the response, with HCl diminishing growth, 2 mM undissociated HL or HAc providing bacteriostatic conditions, that were overcome with prolonged incubation, and with 15 mM undissociated HAc, providing bactericidal conditions. The fact that organic acids display bacteriostatic or bactericidal effects at a given pH, whereas inorganic acids do not, is well-recorded and this has mainly been attributed to a less efficient lowering of the pH_i in the latter case (Brul and Coote, 1999; Cotter and Hill, 2003). However, the physiological effects of different acidulants and their specific induced responses are still unresolved and these topics will be discussed below based on our results obtained with aerobically grown *B. cereus* ATCC 14579.

The phenotypic and transcriptome responses were studied in more detail for the first 60 min of exposure. Differences in viable counts between the growth-inhibited, bacteriostatic and bactericidal conditions were observed. ATP measurements showed that the energy status is maintained within the cells for the non-lethal acid stress conditions, as ATP levels remained constant or were elevated (Fig. 2B). This may be associated with a modulation of oxidative stress response and pyruvate metabolism (Fig. 5, Fig. 6). Under bacteriostatic conditions a more stringent oxidative stress response is observed and additional modulation of amino

acid and oligopeptide transport compared to conditions that diminished growth. Exposure to 15 mM undissociated HAc resulted in inactivation of cells after prolonged exposure and ATP depletion within the first hour. Next to modulation of pyruvate metabolism and oxidative stress response this may be associated with induction of alternative electron transport systems and fatty acid biosynthesis genes. Under non-lethal acid stress conditions, rerouting of the pyruvate metabolism was indicated by induction of a butanediol fermentation pathway and part of the TCA cycle, whereas under bactericidal acid stress conditions lactate, formate, and ethanol fermentation pathways were induced. Apparently, the metabolic rerouting under bactericidal conditions is insufficient to maintain the redox balance and to generate enough ATP.

A prominent aspect of the common acid stress response is the oxidative response. This response is most pronounced for the bacteriostatic conditions, and least pronounced for the bactericidal condition (Fig. 5). This indicates that acid shock of aerobic *B. cereus* ATCC 14579 cells to pH 5.5 may cause increased formation of reactive oxygen species that need to be counteracted as reflected in the increased expression of superoxide dismutase, catalases, and nitric oxide dioxygenase. Induction of superoxide dismutase by *B. cereus* upon acid stress has been reported before (Browne and Dowds, 2002) and an oxidative stress response to mild acid stress has also been reported for *Bacillus subtilis* (Wilks *et al.*, 2009). Notably, the formation of reactive oxygen species and a role for oxidative damage in the bactericidal activity of antibiotics in *Escherichia coli* was recently established (Kohanski *et al.*, 2007). In our work, the induction of the alternative electron-transport chains and NADH-dehydrogenase indicate that the electron transport chain is affected under bactericidal conditions. Similar to the proposed bactericidal mechanism of antibiotics in *E. coli* (Kohanski *et al.*, 2007), induction of oxidative stress related genes may be a response to the formation of reactive oxygen species generated by a perturbation of the electron transport chain. The role of reactive oxygen species in the response of *B. cereus* to acid stress remains to be elucidated and is the subject of further study in our laboratory. The oxidative response in *B. cereus* appears to include the formation of nitric oxide (NO) since genes encoding NO-metabolising enzymes are activated. NO can be produced by nitric oxide synthase (bNOS) and this enzyme has been reported to act as a fast-response protection mechanism in *B. subtilis* and *Bacillus anthracis*. NO, formed from arginine, protects cells from H_2O_2-induced DNA damage by inhibition of the Fenton reaction and activation of catalase (Gusarov and Nudler, 2005; Shatalin *et al.*, 2008). Although bNOS is not differentially expressed, which may be explained by regulation of its function on protein level (Shatalin *et al.*, 2008), orthologues of flavodoxins that support catalysis in *B. subtilis* (e.g., YkuN) are induced under bacteriostatic organic acid stress conditions. These may be responsible for enhanced production of NO from arginine. In addition, Hochgrafe and colleagues (2008) proposed that NO can also protect proteins from irreversible thiol oxidation in *B. subtilis* and *Staphylococcus aureus*. Our data suggest that also in *B. cereus* the endogenous production of NO may provide protection against acid stress.

The bactericidal condition showed increased expression of lactate dehydrogenase (*ldh*) and cytochrome *bd* oxidase (*cydAB*) genes. In *B. subtilis* these genes are co-ordinately expressed together with the lactate permease gene *lctP* and formate-nitrite transporter gene *ywcJ* and under control of the negative regulator YdiH (Rex) (Larsson *et al.*, 2005). Lactate dehydrogenase, which converts lactate to pyruvate, in concert with the cytochrome *bd* oxidase has been proposed to function as an alternative electron transport chain (Chai et al., 2009), which may associate with the oxidative response described above and may additionally contribute to the removal of lactate. Together with the *alsSD* genes, *cydAB*, *ldh*, and *lctP* form a distinct regulon, which is part of the larger Fnr regulon (Reents *et al.*, 2006). The *alsSD* genes encode for enzymes producing acetoin from pyruvate that are involved in anaerobic metabolism (Nakano et al., 1997; Fuchs et al., 2007). This metabolic shift may also result in lower levels of lactate produced from pyruvate. The *B. subtilis alsSD* genes are strongly induced under mild acid stress conditions (Wilks *et al.*, 2009) and the *alsSD* genes in *Lactobacillus plantarum* were shown to contribute to intracellular pH homeostasis (Tsau *et al.*, 1992). In our experiments induction of the *B. cereus alsSD* genes was less pronounced under bactericidal conditions than under the milder stress conditions (Fig. 6). In contrast, *cydAB*, *ldh*, and nitrite transporter gene *ywcJ* were among the highest induced genes upon 15 mM undissociated HAc exposure. In analogy with *B. subtilis*, a changing $NADH/NAD^+$ ratio may be associated with the induced expression of these genes. Adjustments to the cell-envelope (including the cell wall and membrane) may provide most optimal protection of the cell integrity under the different stress conditions, as observed for *L. plantarum* exposed to acid stress conditions (Pieterse *et al.*, 2005) and for *B. subtilis* exposed to sorbic acid stress (ter Beek *et al.*, 2008). The response under bactericidal conditions showed some resemblance with the response of *S. aureus* cells deficit of *murF* that have reduced peptidoglycan synthesis. These cells show down-regulated expression of iron uptake associated genes, induced *ldh*, lactate permease, and formate/nitrite transporter protein genes (Sobral *et al.*, 2007). *B. cereus* cell wall metabolism was mainly affected in the non-lethal acid shock conditions by modulation of teichoic acid and capsular polysaccharide biosynthesis gene expression. Genes involved in lipid biosynthesis were down-regulated under bacteriostatic conditions, which is in line with the reduced need for cell membrane synthesis of the non-growing cells. Under bactericidal conditions however, the fatty acid biosynthesis genes were up-regulated indicating active modulation of the cell membrane under these conditions.

At similar concentrations of undissociated acid, HAc and HL evoke compound-specific responses related to specific actions of the organic acid used and/or differences in the resultant intracellular pH. Expression of a large group of ribosomal protein genes was down-regulated under HAc stress, while moderately up-regulated under HL stress. Several ABC transporters of unknown function were induced under HAc stress conditions, while repressed under HL stress conditions. There was a clear difference in catabolism of branched-chain amino acids, which may result in different adaptations to the lipid composition of the membrane. HAc stress included a shift in sugar PTS systems, and an

apparent switch to anaerobic respiration. HL stress included an up-regulation of glycolysis, TCA cycle and pyruvate metabolism. Rerouting of fermentation products is also observed for *L. plantarum* under lactic acid stress conditions (Pieterse *et al.*, 2005). Up-regulation of genes involved in arginine uptake and metabolism appeared to be HL stress specific. This provides a possibility that *B. cereus* uses arginine conversion to citrulline and ammonia conceivably to counteract HL-induced acidification, which may be linked to the lactic acid-specific up-regulation of the TCA cycle.
In conclusion, we have provided a detailed insight in the different physiological and genetic responses of the food-borne pathogen *B. cereus* to acid shocks set with different acidulants. Acid shock is linked to oxidative stress response and rerouting of pyruvate metabolism. Compared to growth-inhibiting stress conditions, the bacteriostatic conditions evoke a more stringent oxidative stress response involving additional factors involved in redox reactions and conversion of reactive oxygen species. The observed differences in transcriptional responses to HAc and HL exposure may be relevant for their use as food preservatives. The HL-specific response involves fewer genes than the HAc-specific response and in some cases opposite responses were noted, i.e., with genes activated in the presence of HL, whereas they were repressed in the presence of HAc. Under minimal processing conditions these differences could be important in selecting optimal combinations of preservative agents and/or processing conditions to obtain the desired preservation effect.

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Supplementary material
The following supplementary material is available for this chapter online http://www.fhm.wur.nl/uk/thesismaarten: supplementary microarray analyses including tables and figures.

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Chapter 5

Comparative transcriptome and phenotype analysis of acid-stressed *Bacillus cereus* reveals a major oxidative response and inactivation-associated radical formation

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Abstract
Acid stress resistance of the food-borne human pathogen *Bacillus cereus* may contribute to its survival in acidic environments, such as encountered in soil, food, and the human gastrointestinal tract. The acid stress responses of *B. cereus* strains ATCC 14579 and ATCC 10987 were analysed in aerobically grown cultures acidified to pH values ranging from pH 5.4 to pH 4.4 with HCl. Comparative phenotype and transcriptome analyses revealed three acid stress-induced responses in this pH range: growth rate reduction, growth arrest and loss of viability. These phenotypes showed to be associated with metabolic shifts and the induction of general stress response mechanisms with a major oxidative component, including up-regulation of catalases and superoxide dismutases. Flow cytometry analysis in combination with the hydroxyl (OH·) and peroxynitrite ($ONOO^-$) - specific fluorescent probe 3'-(p-hydroxyphenyl) fluorescein (HPF), showed excessive radicals to be formed in both *B. cereus* strains in bactericidal conditions only. Our study indicates that radicals can serve as indicators of acid-induced malfunctioning of cellular processes that lead to cell death.

Introduction

Bacillus cereus is a Gram-positive, spore-forming, facultative anaerobic, rod-shaped food-borne human pathogen that appears to be well-equipped to survive in various adverse conditions. The spores and vegetative cells of *B. cereus* can be found in a range of environments, such as soil (Von Stetten *et al.*, 1999; Vilain *et al.*, 2006), plant rhizosphere (Berg *et al.*, 2005), and various foods (Choma *et al.*, 2000; Rosenquist *et al.*, 2005). Besides being notorious for causing spoilage of dairy products, *B. cereus* is a food-borne pathogen that can cause two distinct types of disease, i.e., emesis and diarrhoea (Kotiranta *et al.*, 2000). The emetic syndrome occurs upon ingestion of the heat-stable toxin cereulide, which is produced in food by emetic *B. cereus* strains (Agata *et al.*, 2002). The diarrheal syndrome is associated with the action of enterotoxins, such as non-haemolytic enterotoxin (NHE) and cytotoxin K (CytK) (Granum and Lund, 1997), that are produced by vegetative cells inside the human small intestine (Stenfors Arnesen *et al.*, 2008). Before entering the small intestine and subsequent production of enterotoxins, *B. cereus* cells have to survive the low pH of the human stomach. Therefore, acid resistance is a key parameter in the pathogenic potential of enterotoxic *B. cereus* strains. Obviously, the highly resistant dormant spores of *B. cereus* can pass the stomach unaffected and germination in the acid environment of the small intestine is an important aspect of their pathogenic potential (Wijnands *et al.*, 2007; Hornstra *et al.*, 2009). Outside the human host, *B. cereus* may also be frequently exposed to acidic conditions including a range of low pH foods, where in specific cases organic acids have been added as preservatives (Brul and Coote, 1999). In conclusion, coping with low pH stress is an important feature in the performance of *B. cereus* in a variety of environments as described above, but also in other ecological niches such as soil and plant rhizosphere (Neumann and Martinoia, 2002).

Acid stress responses have mainly been studied in Gram-negative organisms, such as *Escherichia coli* and *Salmonella* Typhimurium (Richard and Foster, 2003), and in a select number of Gram-positive bacteria, such as lactic acid bacteria and *Listeria monocytogenes* (van de Guchte *et al.*, 2002; Cotter and Hill, 2003; Ryan *et al.*, 2008). These reviews highlight the importance of proton pumps, i.e., F_1F_0-ATPase, transcriptional regulators, such as RpoS (Gram-negatives) and σ^B (Gram-positives), proteins involved in protection of macromolecules, such as DnaK and GroESL, and enzymes that produce alkaline compounds, such as the ammonium forming enzymes urease and arginine deiminase. In contrast, the acid stress response of *B. cereus* has not been studied extensively. Available information is limited to alternative sigma factor σ^B expression upon exposure to a low pH (van Schaik *et al.*, 2004), and the acid tolerance response, which includes modulation of intracellular pH and protein synthesis (Browne and Dowds, 2002; Jobin *et al.*, 2002; Thomassin *et al.*, 2006). Additionally, the role of urease in acid resistance of a large number of *B. cereus* strains has been studied (Mols and Abee, 2008), and revealed that its role in acid resistance of *B. cereus* was limited.

Therefore, we set out to investigate the molecular mechanisms involved in acid stress response of *B. cereus* and to identify possible acid-induced inactivation mechanisms, by

comparing responses of cells exposed to selected pHs leading to mild, bacteriostatic and bactericidal acid stress. To determine both general and phenotype-associated transcriptional responses, two model strains ATCC 14579, isolated from air (Ivanova *et al.*, 2003) and ATCC 10987, a food-isolate (Rasko *et al.*, 2004) were investigated. Recently, Kohanski and colleagues (2007) reported that the formation of reactive oxygen species (ROS), such as hydroxyl radicals (OH·), plays a role in antibiotic-induced inactivation of aerobically grown *E. coli* and *Staphylococcus aureus* cells. These ROS were suggested to originate from antibiotic-induced perturbation of the electron transfer chain resulting in the production of superoxide (O_2^-). O_2^- can damage iron-sulphur clusters and subsequently react with the released iron, resulting in OH· formation via the Fenton reaction. Therefore, flow cytometry analysis, in combination with the OH· and peroxynitrite ($ONOO^-$) -specific fluorescent probe 3'-(p-hydroxyphenyl) fluorescein (HPF) (Setsukinai *et al.*, 2003), was included in our study to detect ROS in (sub)lethally acid-stressed *B. cereus* cells. Our study provides evidence that radicals can serve as indicators of acid-induced malfunctioning of cellular processes and the stress-induced formation of reactive oxygen species as a common theme in bacterial stress response and cellular death is discussed.

Materials and methods

Bacterial strains and growth conditions

B. cereus strains ATCC 14579 and ATCC 10987 were obtained from the American Type Culture Collection (ATCC). Stock cultures, grown in brain heart infusion (BHI, Becton Dickinson, France) broth, were stored at -80°C in 33% glycerol. To prepare pre-cultures, 10 ml BHI in a 100 ml Erlenmeyer flask was inoculated with a droplet from the glycerol stock and incubated overnight at 30°C, with shaking at 200 rpm.

To study the effect of pH on *B. cereus* cells and the corresponding transcriptome profiles, 100 ml BHI in a 500 ml Erlenmeyer flask was inoculated with 0.5 ml pre-culture and incubated at 30°C, with shaking at 200 rpm. Upon reaching an optical density of 0.5 measured at 600 nm (OD, Novaspec II, Pharmacia Biotech, Germany), the culture pH was measured (PHM 240 pH/ION Meter, Radiometer, Denmark) and serial dilutions were made in peptone physiological salt solution (PPS, 1g/l neutralized bacteriological peptone (Oxoid, England) and 8.5 g/l NaCl in water) and plated with a spiral-plater (Eddy Jet; IUL Instruments, Spain) on BHI agar plates (15 g/l bacteriological agar, Oxoid, England). 20 ml of the culture was used to extract RNA (sample t = 0). The remaining volume of the culture was acidified with hydrochloric acid (HCl 37%, Merck, Germany) to pH 5.4, 5.0, 4.8 or 4.5 and incubated at 30°C, with shaking at 200 rpm. At designated time points (10, 30 and 60 minutes), samples were taken to measure the OD, to determine the viable counts, and to extract RNA.

RNA isolation

RNA isolation was performed by transferring 20 ml of the cultures into a 50-ml Falcon tube (Greiner Bio-one, Germany) at the designated time points. Subsequently, the cultures were pelleted at maximum speed at 4°C for 30 s (Eppendorf centrifuge 5804 R, Eppendorf, Germany). After decanting the supernatant, the cell pellets were frozen in liquid nitrogen. Within 10 min after freezing the cell pellets, 1 ml TRI-reagent (Ambion, United Kingdom) was added to the pellets. The samples were stored at -80°C until RNA extraction. RNA was extracted as described previously (van Schaik *et al.*, 2004). Residual chromosomal DNA was removed by treating the samples with DNA-*free* (Ambion, United Kingdom). The RNA concentration was measured in 2 ml cuvettes (UVettes, Eppendorf, Germany) with a BioPhotometer (Eppendorf, Germany) by determining the OD_{260} and OD_{280}. The quality of the RNA was monitored using the RNA 6000 Nano Assay (Agilent, United States) and the Agilent 2100 Bio-analyzer (Agilent, United States) according to the provided protocol. The extracted RNA samples were stored in 70% ethanol with 83 mM sodium acetate buffer (pH 5.2) at -20°C.

cDNA synthesis, labelling and microarray hybridization and design

Complementary DNA with amino-allyl-labelled dUTP (Ambion, United Kingdom) from the extracted RNA was prepared in reverse transcription reactions using Superscript III (Invitrogen, The Netherlands). Cy3 and Cy5 labelling of the cDNAs was performed with the CyScribe Post-Labeling kit (GE Healthcare, Belgium) as previously described (den Hengst *et al.*, 2005). The labelled cDNAs were purified using the CyScribe GFX purification kit (GE Healthcare) according to the provided protocol. To conduct the microarray hybridization, the Cy5-labelled cDNA samples were combined with the corresponding Cy3-labelled t0 reference samples (1:1 ratio). The microarray experiments for the comparison of the transcriptomes of the cultures exposed to various pHs were performed in two independent biological replicates, where the replicate was performed with the dyes swapped. *B. cereus* ATCC 14579 and *B. cereus* ATCC 10987 microarrays (details below) were hybridized with 200 to 300 ng labelled cDNA following the 60-mer oligo microarray processing protocol (Agilent, United States).

The microarrays used in this study were custom-made *B. cereus* ATCC 14579 and custom-made *B. cereus* ATCC 10987 microarrays developed by Agilent Technologies (United States). The *B. cereus* ATCC 14579 microarray design was based on the 11K platform of Agilent Technologies (GEO accession number GPL7680). A total of 10,262 spots represented 5,131 chromosomal open reading frames, meaning that 98.0% of the predicted chromosomal open reading frames (NCBI accession number NC_004722) are represented on the microarray. 99.6 % of the open reading frames for which probes could be designed were represented by two non-overlapping probes on the array. The remaining 0.4% of the open reading frames was represented by a single oligonucleotide spotted in duplicate on the array. The *B. cereus* ATCC 10987 microarray design was based on the 22K platform of Agilent Technologies (GEO accession number GPL7681). A total of 17,697 spots

represented 5,578 chromosomal open reading frames, 240 plasmid open reading frames and 81 putative small-RNAs, meaning that 99.6% of the predicted chromosomal and the plasmid open reading frames (NCBI accession numbers NC_003909 and NC_005707, respectively) were represented on the microarray. All features (chromosomal and plasmid open reading frames and small-RNAs) were represented by three individual spots. For 4,914 features three non-overlapping probes were designed, for 488 features two probes were designed (one probe spotted in duplicate) and for 497 only one oligonucleotide could be designed (one probe spotted in triplicate).
After hybridization at 60°C for 17 hours, the microarrays were washed with 6 × SSC (0.9 M NaCl and 0.09 M sodium citrate) supplemented with 0.005% Triton X-102 at room temperature for 10 min. Subsequently, the microarray slides were washed at 4°C with prechilled 0.1 × SSC with 0.005% Triton X-102 for 5 min and dried with nitrogen gas.

Microarray scanning and data analysis
The microarray slides were scanned using an Agilent microarray scanner (G2565BA), and data were extracted from the scanned microarrays with Agilent's Feature Extraction software (version 8.1.1.1), which includes a LOWESS (locally weighted scatterplot smoothing) normalization step for the raw data. After removal of the data for the control spots, the normalized data for each spot from the microarrays were analyzed for statistical significance using the web-based VAMPIRE microarray suite (Hsiao *et al.*, 2005). A spot was found to be differentially expressed between two samples when the false discovery rate was smaller than 0.05. Subsequently, the data for the single spots were integrated to obtain expression ratios for a corresponding feature (i.e., open reading frame or sRNA). A feature was found to be differentially expressed when all spots representing the feature were significantly differentially expressed between samples.
Hierarchical clustering (Eisen *et al.*, 1998) was performed per strain to identify groups of genes showing similar expression patterns. In Genemaths XT (version 1.6.1, Applied Maths, Belgium) genes that were significantly differentially expressed in one or more conditions were $\log_2$ transformed and clustered using the complete linkage method and the Euclidian distance matrix. The groups identified from the hierarchical clustering were based on an arbitrary cut-off value. To identify relevant biological processes significantly overrepresented in a group, the genes corresponding to a group were analyzed using FIVA (Blom *et al.*, 2007). To visualize the relation between the different acidic conditions independent of the strains, the $\log_2$ transformed expression ratios from genes present on both microarrays were hierarchically clustered using the average linkage method and the Euclidian distance matrix (Genemaths XT, 1.6.1).

Flow cytometry and radical measurements
To detect radical formation, the fluorescent reporter dye 3'-(p-hydroxyphenyl) fluorescein (HPF, Invitrogen, The Netherlands) was used (Setsukinai *et al.*, 2003). At designated time points (0, 10, 30, 60 minutes) after adding HCl to the exponentially growing cultures

(OD_{600nm} ~0.5), samples were obtained by centrifuging (15,000 × *g*, 30 s) 1 ml of culture and resuspension of the cell pellet in 1 ml filtered phosphate buffered saline (PBS). The samples were washed once and diluted with filtered PBS to obtain a concentration of approximately 10^6 cells per ml, subsequently HPF was added at a final concentration of 5 mM. Samples were run on a Becton Dickinson FACSCalibur flow cytometer with the following photomultiplier tube (PMT) voltage settings: E00 (FSC), 360 (SSC) and 825 (FL1). Data were obtained from 20,000 events (cells) at medium flow rate using Cellquest Pro (version 4.0.2), subsequently analyzed with WinMDI 2.9 (Joseph Trotter, Salk Institute for Biological Studies, La Jolla, California, USA; http://facs.scripps.edu/software.html) and graphically presented using Adobe Illustrator CS2 (version 12.0.1).

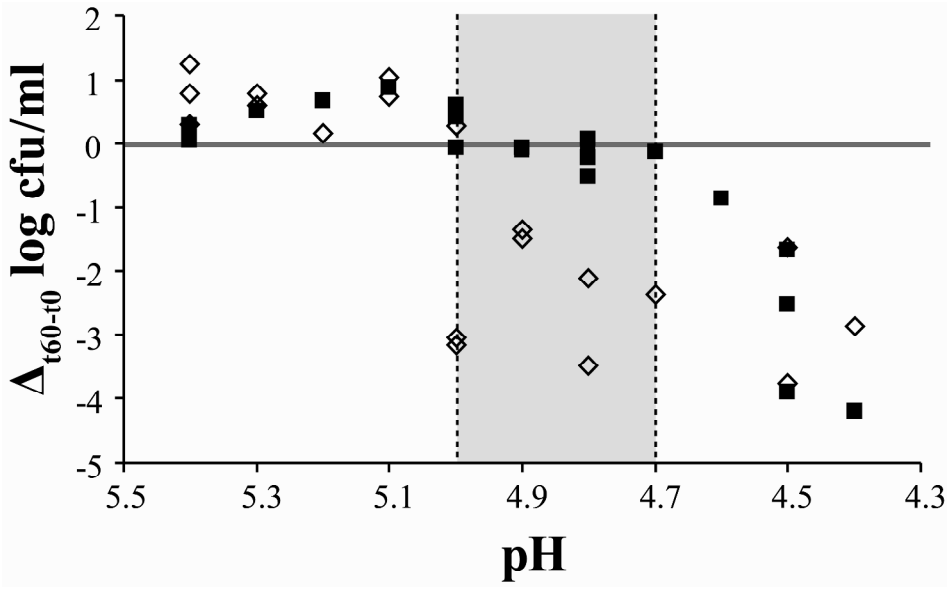

Fig. 1. Physiological response of *B. cereus* ATCC 14579 (filled squares) and ATCC 10987 (open diamonds) upon exposure to a range of acidity levels. The colony forming units were determined after 0 and 60 minutes of exposure, the difference between the t0 and t60 is depicted. The grey line at 0 corresponds to no growth (growth above the line) and no inactivation (inactivation below the line). The filled area between the dotted lines depicts the pH values where ATCC 14579 displayed no growth and no inactivation, i.e., survival, and where ATCC 10987 already showed to be inactivated.

Results

Physiological response to acid stress

The physiological response to acid stress was studied using *B. cereus* strains ATCC 14579 and ATCC 10987 by acidifying aerobically grown cultures to pH values ranging from pH

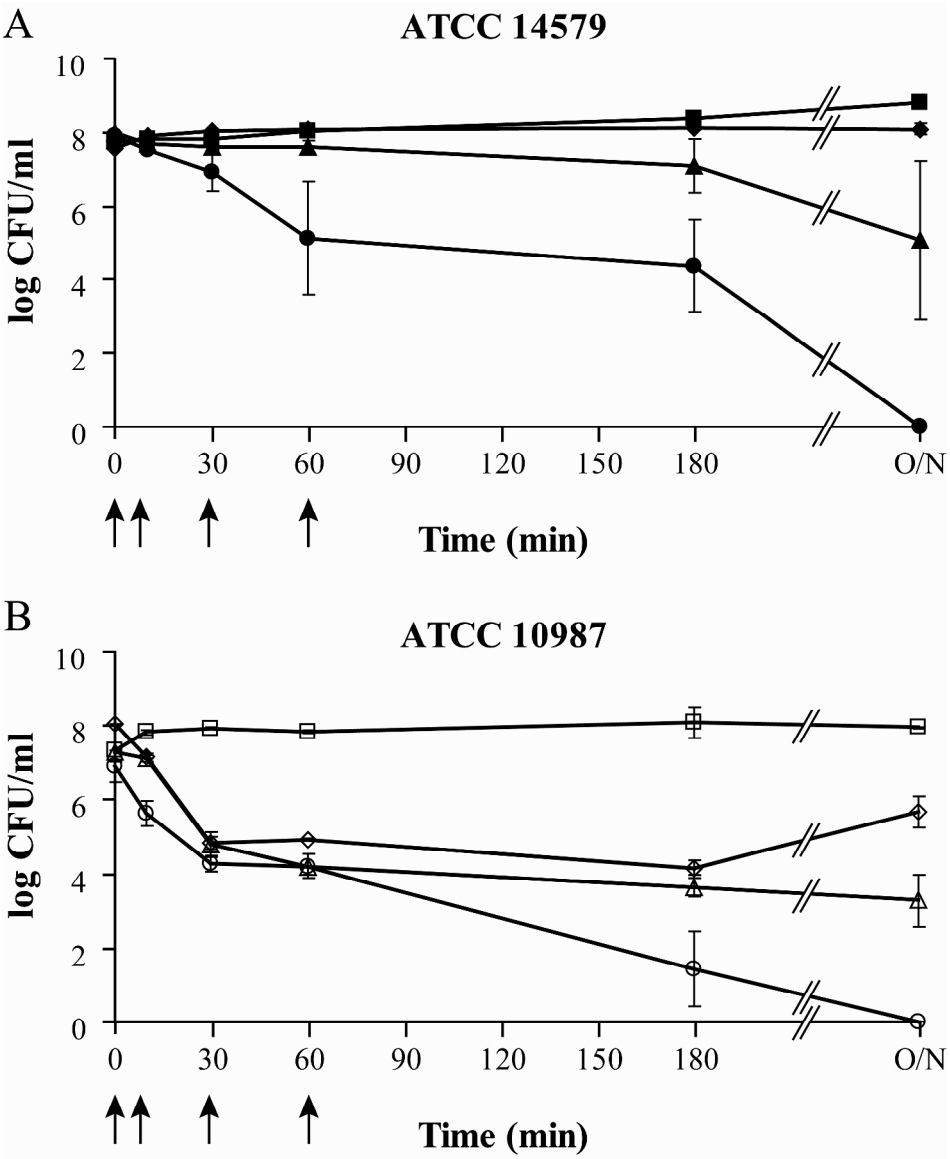

Fig. 2. Physiological response of *B. cereus* ATCC 14579 (A) and ATCC 10987 (B) upon exposure to low pH. The colony forming units determined at different time points upon exposure to pH 5.4 (squares), pH 5.0 (diamonds), pH 4.8 (triangles) and pH 4.5 (circles) are depicted. At 0, 10, 30 and 60 minutes samples were taken for microarray analysis, indicated with arrows and the error bars represent the standard deviation between duplicate experiments.

5.4 to pH 4.4 by addition of HCl (Fig. 1). Upon exposure to the different acid shocks, the growth of exponentially growing *B. cereus* cells was instantly affected. The two strains used showed different phenotypic responses to different levels of acidity. *B. cereus* strain ATCC 14579 showed to continue growth as reflected in an increase of colony forming units upon exposure to pH shocks as low as pH 5.0. This response is hereafter referred to as growth phenotype. Strain ATCC 14579 was inactivated at pH 4.6 and lower as shown by the inability to form colonies on BHI plates incubated at 30°C for 16 hours. This response is hereafter referred to as inactivation phenotype and the condition as bactericidal. Upon exposure to pHs between pH 5.0 and 4.7, ATCC 14579 showed a stable number of viable counts within the first hour of exposure. This response is hereafter referred to as survival phenotype and the condition as bacteriostatic. However, prolonged exposure (overnight) resulted in a decrease of viable counts (Fig. 2). The growth boundary of the other strain tested, *B. cereus* ATCC 10987, was determined at pH 5.0. Upon exposure to pHs higher than pH 5.0, ATCC 10987 was able to grow and at pHs lower than pH 5.0 this strain was inactivated. There was no apparent survival phenotype in the ATCC 10987 acid shock response as observed for ATCC 14579 between pH 5.0 and pH 4.7. The display of an intermediate physiological survival response by strain ATCC 14579 and the different inactivation boundaries were the main differences between the two strains tested.

Microarray analysis using hierarchical clustering

To investigate the impact of mild, bacteriostatic and bactericidal acid shocks on the gene expression of the two *B. cereus* strains, four pHs were selected based on the different phenotypic responses displayed by the two strains (Fig. 2A and 2B). At 0, 10, 30 and 60 minutes after the exposure to the different pH shocks, i.e., pH 5.4, pH 5.0, pH 4.8 and pH 4.5, RNA samples were collected and subsequent microarray analyses were performed. To compare the transcriptomes of both strains, data obtained of orthologous genes that are present in the genomes of both ATCC 14579 and ATCC 10987 (Ivanova *et al.*, 2003; Rasko *et al.*, 2004; Mols *et al.*, 2007) were collected and subjected to hierarchical clustering. The transcriptome profiles clustered in two different groups, with one cluster including samples obtained of the growth phenotype and the other cluster encompassing samples of the survival (strain ATCC 14579 only) and inactivation phenotypes (Fig. 3). The transcriptome profiles of cultures that showed growth after acid shock exposure clustered together independent of the exposure time and strain. Within these two major groups, the different branches of the hierarchical clustering were separated mostly depending on strain and exposure pH rather than exposure time. In conclusion, the exposure of *B. cereus* ATCC 14579 and ATCC 10987 to mild, bacteriostatic and bactericidal acid stress led to phenotype specific transcriptome profiles independent of exposure time.

The pH- and phenotype-specific responses were investigated by analyzing the transcriptome data obtained for genes showing significant differential expression in one or more conditions per strain. Groups of genes with similar expression profiles were identified

using hierarchical clustering (*Supplementary material*). The results obtained for a selection of genes putatively involved in low pH or oxidative responses are presented and discussed below.

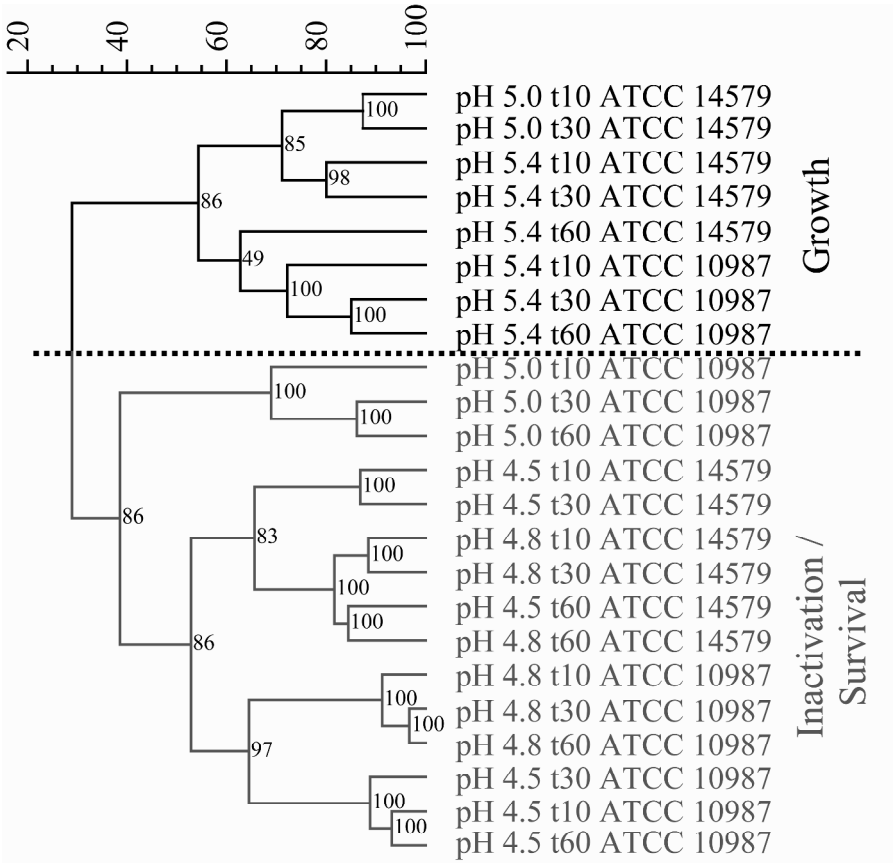

Fig. 3. Hierarchical clustering of the transcriptome profiles of different pH exposures based on the common genes of *B. cereus* ATCC 14579 and ATCC 10987. Samples with similar expression patterns were clustered using Euclidean distance and complete linkage. The corresponding phenotypic responses are shown at the right. Samples obtained from growing cultures are depicted in black, samples from non-growing ("survival" and "inactivation" phenotypes) cultures are shown in grey. Relative distance in similarity between the branches is shown at the top and bootstrap values are indicated at each branch.

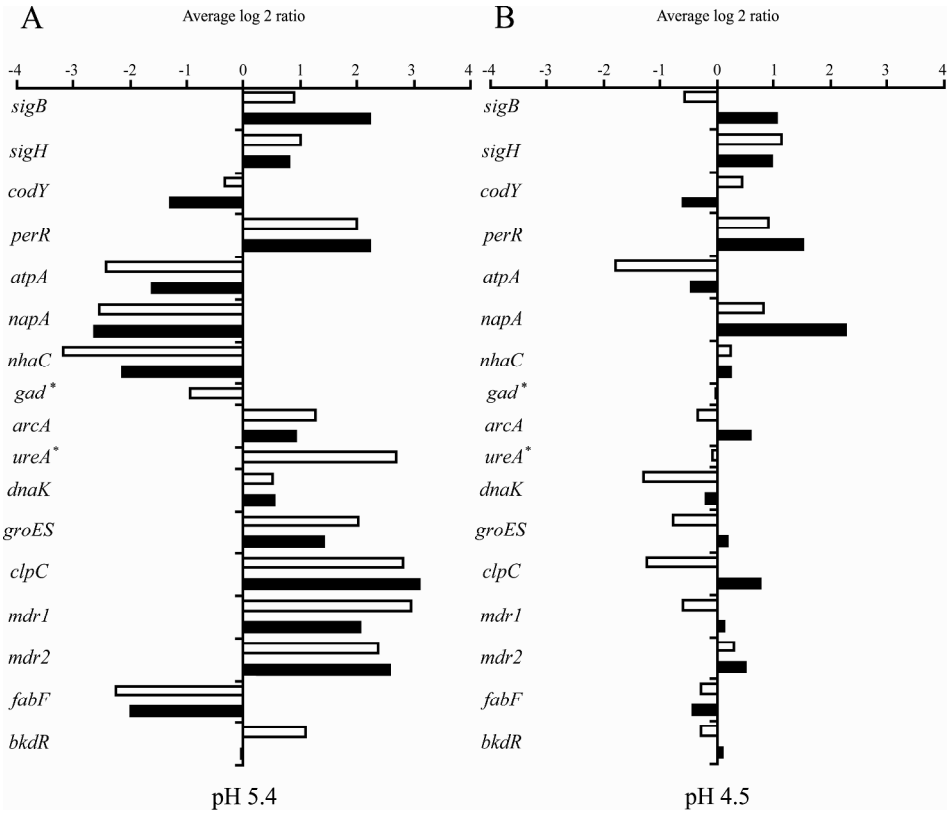

Fig. 4. Average ratios of low pH and stress associated genes from *B. cereus* ATCC 14579 (closed bars) and ATCC 10987 (open bars) upon exposure to pH 5.4 (A) and pH 4.5 (B). The global adaptive response is represented by *sigB* (BCE1086 and BC1004), *sigH* (BCE0093 and BC0114) and *codY* (BCE3869 and BC3826), encoding σ^B, σ^H and CodY respectively. Additionally, *perR* (BCE0592 and BC0518), a major oxidative stress response regulator is shown. *atpA* (BCE5432 and BC5308), *napA* (BCE1729 and BC1612), and *nhaC* (BCE1840 and BC1709) represent F_1F_0-ATPase and two proton antiporters. *gad* (glutamate decarboxylase, BCE2691), *arcA* (arginine deiminase, BCE0472 and BC0406), and *ureA* (urease, BCE3664) represent systems that are described to be involved in alkaline production. Glutamate decarboxylase and urease are specific for ATCC 10987 and are indicated with an asterisk. The general stress response chaperones and proteases are depicted by *dnaK* (BCE4395 and BC4312), *groES* (BCE0288 and BC0294), and *clpC* (BCE0079 and BC BC0100). Additionally, mechanisms involved in sorbic acid stress of *B. subtilis* (Ter Beek *et al.*, 2008), such as, multidrug transporters (*mdr1*, BCE4699 and BC4568 and *mdr2*, BCE1943 and BC1786), fatty acid biosynthesis (*fabF*, BCE1294 and BC1174), and branched-chain fatty acid biosynthesis (*bkdR*, BCE4239 and BC4165) are shown.

Acid shock response of low pH associated genes

A selection of genes, based on their putative role in acid stress response of other Gram-positive organisms (Cotter and Hill, 2003; Ter Beek *et al.*, 2008), was monitored profoundly. The selection includes transcription regulators, proton pumps, glutamate decarboxylase, production of alkaline compounds, protection of macromolecules, membrane synthesis, and multidrug transporters. The ratios of these genes obtained from cells exposed to pH 5.4 and pH 4.5 for 10, 30 and 60 minutes were averaged and plotted per gene (Fig. 4). In general, the average ratios, showing the up- or down-regulation, were less pronounced in inactivated cells. The genes encoding sigma factors σ^B and σ^H, involved in the global adaptive response to stress, were slightly up-regulated in growing cells of both strains. On the other hand, *codY*, which is a key regulator in the nutrient starvation response of Gram-positive organisms, showed no significant up-regulation. The major oxidative stress response regulator *perR* was one of the most up-regulated transcription regulators in both growing and inactivated cells, indicating an oxidative response upon low pH exposure. Previously, proton pumps, i.e., F_1F_0-ATPase, were shown to contribute to pH homeostasis in fermenting Gram-positives exposed to mild acid conditions (Cotter and Hill, 2003). In this study, genes encoding subunits of the F_1F_0-ATPase (represented by *atpA* in Fig. 4) were highly down-regulated in aerobically grown and exposed *B. cereus* cells upon exposure to sub-lethal pHs. Upon exposure to lethal acid shocks, genes encoding sodium-proton antiporters *napA* and *nhaC* were not down-regulated and *napA* even showed to be up-regulated. In *Listeria monocytogenes* (Cotter *et al.*, 2001) and *Lactococcus lactis* (Sanders *et al.*, 1998) acid-induced glutamate decarboxylase (*gad*), which catalyzes the decarboxylation of glutamate with concomitant consumption of protons, was found to play an important role in low pH survival. In *B. cereus* ATCC 10987, however, the *gad* gene, that is not present in the genome of ATCC 14579, showed not to be up-regulated upon low pH exposure. This is in line with the notion that *B. cereus* ATCC 10987 lacks a glutamate/GABA exchanger (Mols *et al.*, 2007), that is required to supply glutamate decarboxylase with its substrate (Cotter and Hill, 2003). Alkaline compound forming mechanisms, such as the arginine deiminase (ADI) pathway and the urease enzyme, are involved in acid tolerance of Gram-positive organisms (Cotter and Hill, 2003). Arginine deiminase (*arcA*), which is involved in acid resistance of streptococci (Curran *et al.*, 1995) and *L. monocytogenes* (Ryan *et al.*, 2009), showed significant up-regulation in both *B. cereus* strains upon exposure to sub-lethal acid shocks, whereas exposure to bactericidal acid shocks revealed no significant induction. Urease encoding genes, specific for ATCC 10987 (Mols *et al.*, 2007), were induced upon exposure to pH 5.4 (represented by *ureA* in Fig. 4A and 4B), but not in bactericidal conditions. Macromolecules are easily damaged during stress exposure, and their protection and repair is crucial for bacterial survival. DnaK and GroES are chaperones, preventing misfolding of proteins, and in *Streptococcus mutans* deletion of these chaperones resulted in less resistant cells (Lemos *et al.*, 2001). Notably, chaperone encoding genes *dnaK* and *groES* and protease encoding gene *clpC* were

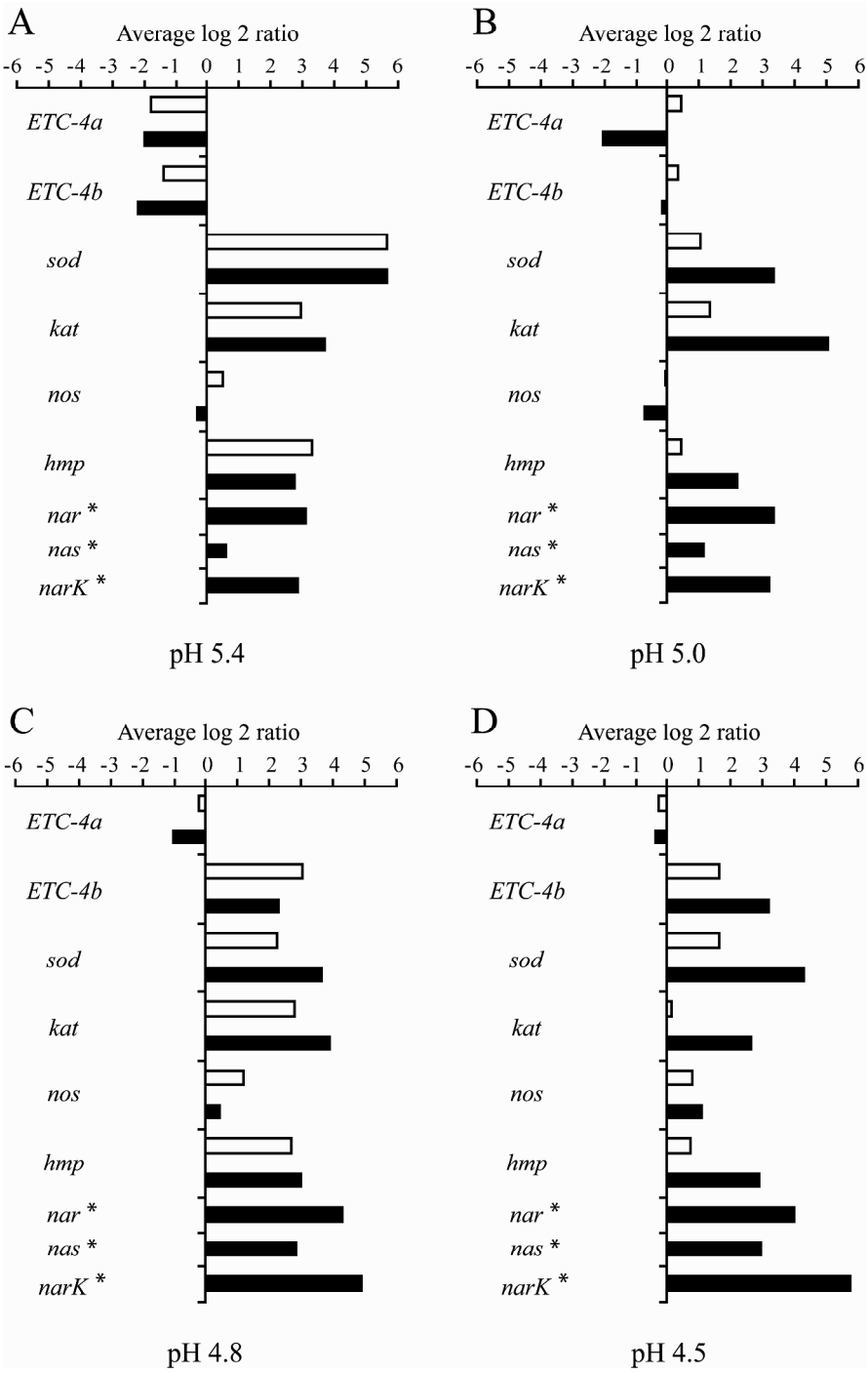
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Fig. 5. Average ratios of selected genes associated with respiration and oxidative responses from *B. cereus* ATCC 14579 (closed bars) and ATCC 10987 (open bars) upon exposure to pH 5.4 (A), pH 5.0 (B), pH 4.8 (C) and pH 4.5 (D). *ETC-4a* and *ETC-4b* depict two distinct types of complex IV of the aerobic electron transfer chain. Cytochrome C oxidase polypeptide I gene *ctaD* (BCE3990 and BC3943) represents *ETC-4a* and cytochrome D ubiquinol oxidase subunit I gene *cydA* (BCE4949 and BC4792) represent *ETC-4b*. The genomes of ATCC 14579 and ATCC 10987 harbour four different superoxide dismutase genes and three different catalase genes, the data of *sodA* (BCE5579 and BC5445) and *katB* (BCE1261 and BC1155) are shown here. Nitric oxide synthase and nitric oxide dioxygenase are represented by the corresponding genes, *nos* (BCE5578 and BC5444) and *hmp* (BCE1571 and BC1448), respectively. Nitrate and nitrite reductases are encoded by multiple genes on the genome of ATCC 14579, therefore *narI* (BC2121), *nasD* (BC2136) and *nark* (BC2128) are shown. This nitrate and nitrite reductase cluster of ATCC 14579 is absent in ATCC 10987 and therefore indicated with an asterisk.

up-regulated upon exposure to sub-lethal acid shocks, whereas exposure to lethal pHs did not induce these genes in *B. cereus*.
Mild sorbic acid stress induces the expression of the fatty acid biosynthesis genes (*fab*), *bkdR* and a multidrug transport gene in *B. subtilis* (Ter Beek *et al.*, 2008). Two homologous multidrug systems (*mdr1* and *mdr2*) showed to be also up-regulated in sub-lethal inorganic acid stress in *B. cereus*. In contrast to sorbic acid stressed *B. subtilis*, fatty acid biosynthesis (represented by *fabF* in Fig. 4A and 4B) was down-regulated at pH 5.4 and no significant induction was found for branched-chain fatty acid biosynthesis (*bkdR*) in *B. cereus* upon exposure to (sub)lethal inorganic acid stress.

Oxidative response and rearrangements in energy metabolism
The response of several genes involved in oxidative stress and energy production were investigated in more detail (Fig. 5). Two distinct types of cytochrome oxidases showed different expression patterns. Cytochrome C oxidase, which acts as complex IV in aerobic conditions, was repressed in sub-lethal conditions in both strains. Cytochrome D ubiquinol oxidase, which can act as an alternative complex IV, was also down-regulated upon exposure to pH 5.4. However, it was highly induced in bacteriostatic and bactericidal conditions. Genes involved in oxidative stress, such as *sodA*, *katB* (Fig. 5) and *perR* (Fig. 4) were highly up-regulated in all acid shock conditions tested. The induction of these genes indicates that a low pH may induce the formation of oxidative compounds, such as H_2O_2. Nitric oxide (NO), formed from arginine by nitric oxide synthase (*nos*), putatively protects cells from H_2O_2-induced DNA damage by inhibition of the Fenton reaction and activation of catalase (Gusarov and Nudler, 2005; Shatalin *et al.*, 2008). Although *nos* was only slightly up-regulated in bactericidal conditions, the formation of nitric oxide may be inferred from the induction of nitric oxide dioxygenase (*hmp*) and a nitric oxide dependant transcriptional regulator (*dnrN*, *Supplementary material*). Nitric oxide dioxygenase facilitates the reaction of nitric oxide with oxygen to form nitrate. Nitrate reductase (*nar*) and nitrite reductase (*nas*) are involved in nitrogen metabolism and may serve as an alternative for aerobic respiration. Nitrate reductase and nitrite reductase genes are unique

for strain ATCC 14579 and cluster together with nitrite extrusion protein *narK* (Mols *et al.*, 2007). The cluster, including *nar*, *nas* and *narK*, was highly up-regulated upon exposure to all acid shocks tested.

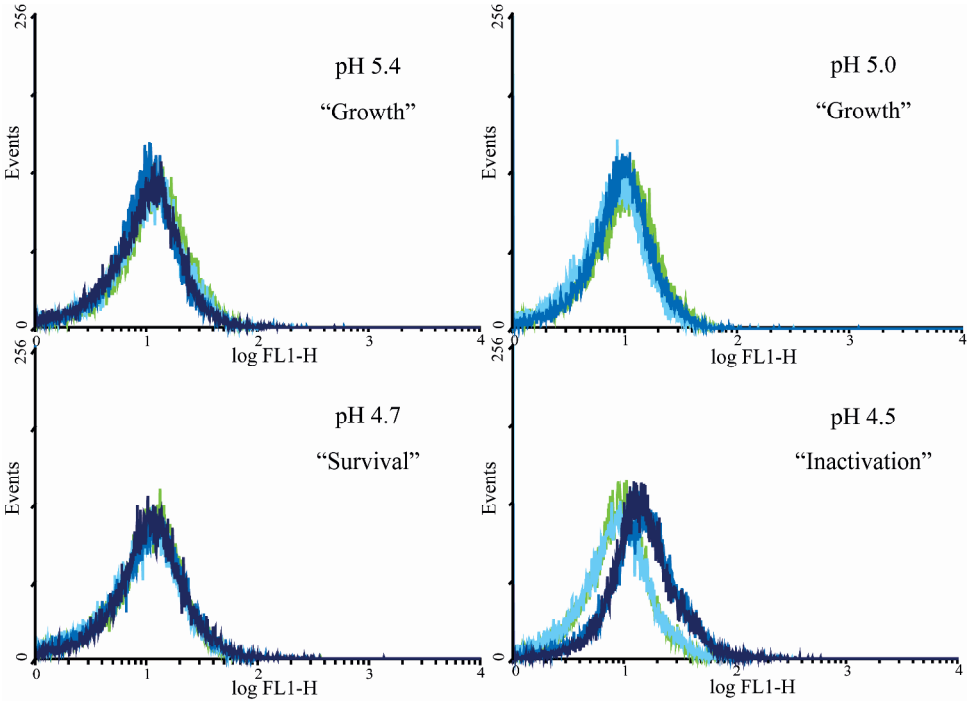

Fig. 6. Radical formation in *B. cereus* ATCC 14579 upon exposure to pH 5.4, pH 5.0, pH 4.8 and 4.5. Samples were taken at 0 (green), 10 (light blue), 30 (blue) and 60 (dark blue) minutes. The pH and corresponding physiological response are indicated at each graph. The shift in fluorescent signal to the right indicates the formation of hydroxyl and/or peroxynitrite radicals.

Inactivation associated radical formation

The induction of oxidative stress associated genes and a recent publication that showed that hydroxyl radicals (OH·) were formed upon exposure to bactericidal antibiotics in *Escherichia coli* and *Staphylococcus aureus* (Kohanski *et al.*, 2007), prompted us to investigate the formation of radicals upon low pH exposure of *B. cereus*. The formation of OH· and/or peroxynitrite ($ONOO^-$) in ATCC 14579 and ATCC 10987 cells was tested upon exposure to selected pHs (pH 5.4, pH 5.0, pH 4.8, and pH 4.5) at different intervals using the fluorescent probe 3'-(p-hydroxyphenyl) fluorescein (Fig. 6 and Fig. 7, respectively). Upon exposure to pH 4.5, ATCC 14579 was inactivated and this pH induced an increase of fluorescence indicating the formation of OH· and/or $ONOO^-$. The exposure to the other pHs tested, i.e., pH 5.4, 5.0, and 4.8, did not result in inactivation of the cells and also did not

induce excess radical formation. Strain ATCC 10987 showed excess radical formation corresponding to the inactivation observed at pH 5.0, pH 4.8 and pH 4.5. At pH 5.4, where this strain was able to resume growth, no excess radical formation was measured. To conclude, the formation of hydroxyl radicals and/or peroxynitrite was associated with inactivation of *B. cereus* strains ATCC 14579 and ATCC 10987 exposed to low pH environments.

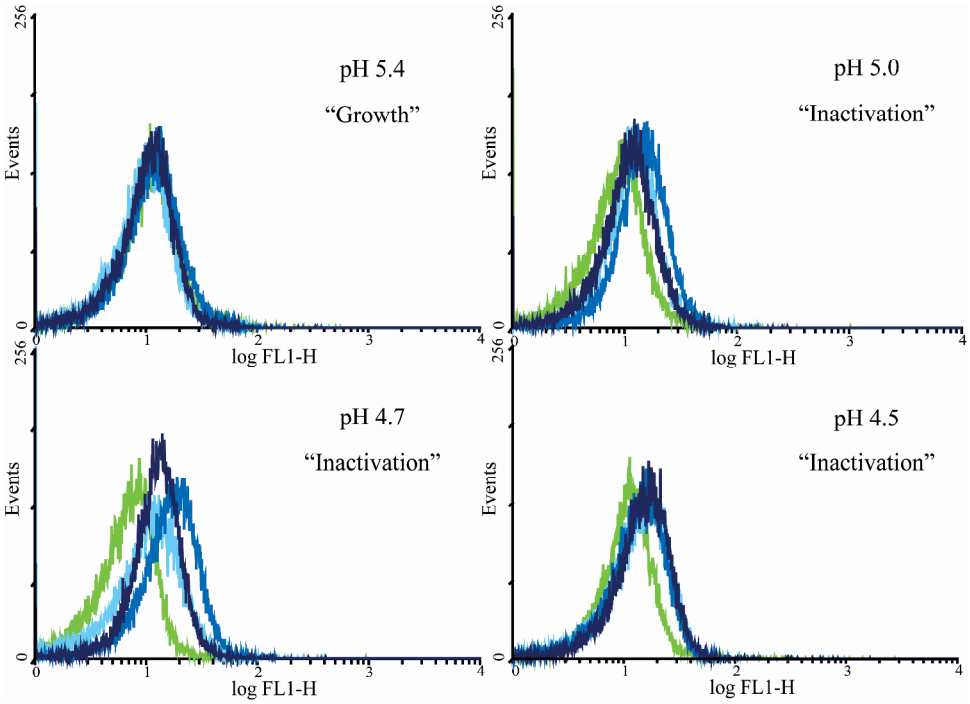

Fig. 7. Radical formation in *B. cereus* ATCC 10987 upon exposure to pH 5.4, pH 5.0, pH 4.8 and 4.5. Samples were taken at 0 (green), 10 (light blue), 30 (blue) and 60 (dark blue) minutes. The pH and corresponding physiological response are indicated at each graph. The shift in fluorescent signal to the right indicates the formation of hydroxyl and/or peroxynitrite radicals.

Discussion

In this study, we describe the physiological and transcriptional responses of *Bacillus cereus* strains ATCC 14579 and ATCC 10987 to sub-lethal and lethal acid shocks. The two model strains were subjected to a range of pHs demonstrating that ATCC 14579 was more acid-resistant than ATCC 10987. ATCC 14579 survived acid conditions between pH 5.0 and pH 4.7 without growth or inactivation in the first hour of exposure. However, a prolonged exposure of ATCC 14579 to pH 4.8 resulted in a decrease of viable cells. In contrast,

ATCC 10987 did not display this survival phenotype and was inactivated already within the first hour of exposure to pHs lower than pH 5.0.

The exposure of *B. cereus* to sub-lethal and lethal acid stress resulted in distinct transcriptome profiles related to the physiological response displayed by the cultures. The concurrent analysis of two strains thus enables for distinguishing between phenotype-specific, stress level-specific and strain-specific transcriptome responses. Furthermore, the approach used showed not only the well-studied responses to mild pHs, including the induction of several general stress response genes, but also the response to lethal levels of acidity, an issue that has up to now mostly been neglected, as exemplified in recent studies on mild acid stress response of *Bacillus subtilis* (Wilks *et al.*, 2009). Cotter and Hill (2003) have reviewed the response of Gram-positive organisms to mild levels of acidity and mechanisms of acid resistance were described for fermentative lactic acid bacteria and *L. monocytogenes*, including roles of proton pumps, regulators, altered metabolism, protein and DNA repair, cell envelope alterations and alkali production. Using two model strains of *B. cereus*, we have demonstrated that protein and DNA repair, stress related transcriptional regulators, altered metabolism and alkali production were indeed induced at low pH. In contrast to fermentative lactic acid bacteria, F_1F_0-ATPase was not up-regulated in these respiring *B. cereus* strains upon exposure to acid, indicating that *B. cereus* does not use F_1F_0-ATPase to extrude protons under the conditions tested. Down-regulation of F_1F_0-ATPase is best explained by the cells trying to prevent excessive inward flux of protons via this ATPase upon exposure to acid conditions. Furthermore, no indications were found in the transcriptome analyses for low pH-induced membrane damage or rearrangement of membrane composition. For example, our experiments did not show an induction of fatty acid biosynthesis (*fab* genes), as was shown for *B. subtilis* exposed to mild sorbic acid stress (Ter Beek *et al.*, 2008). Furthermore, Ter Beek and colleagues (2008) reported that a putative multidrug resistance (mdr) transporter was induced in *B. subtilis* exposed to mild sorbic acid stress and they proposed this transporter to export sorbate anions from the cell. Two homologous genes in *B. cereus* were up-regulated upon exposure to inorganic acid stress at pH 5.4 that were not induced in response to lethal pH exposures. Since there is no apparent connection with sorbic acid stress and the induction of these putative mdr systems in *B. cereus*, their role in acid resistance of *B. cereus*, if any, remains to be elucidated.

The transcriptome analyses of the phenotypic responses to various levels of acidity revealed a major oxidative response. In bactericidal conditions, the oxidative response could be linked to the formation of OH· and/or $ONOO^-$ using flow cytometry in combination with the fluorescent probe HPF that specifically targets these reactive oxygen species. The observed oxidative burst in *B. cereus* may originate in a similar way as described for the formation of OH· radicals in *Escherichia coli* and *Staphylococcus aureus* upon exposure to bactericidal antibiotics (Kohanski *et al.*, 2007). Based on phenotype and transcriptome analyses we propose a model for acid-induced radical formation, including OH· and $ONOO^-$, in *B. cereus* (Fig. 8). Acid stress may cause perturbation of the aerobic electron transfer chain (ETC) in *B. cereus* indicated by the differential expression of several genes

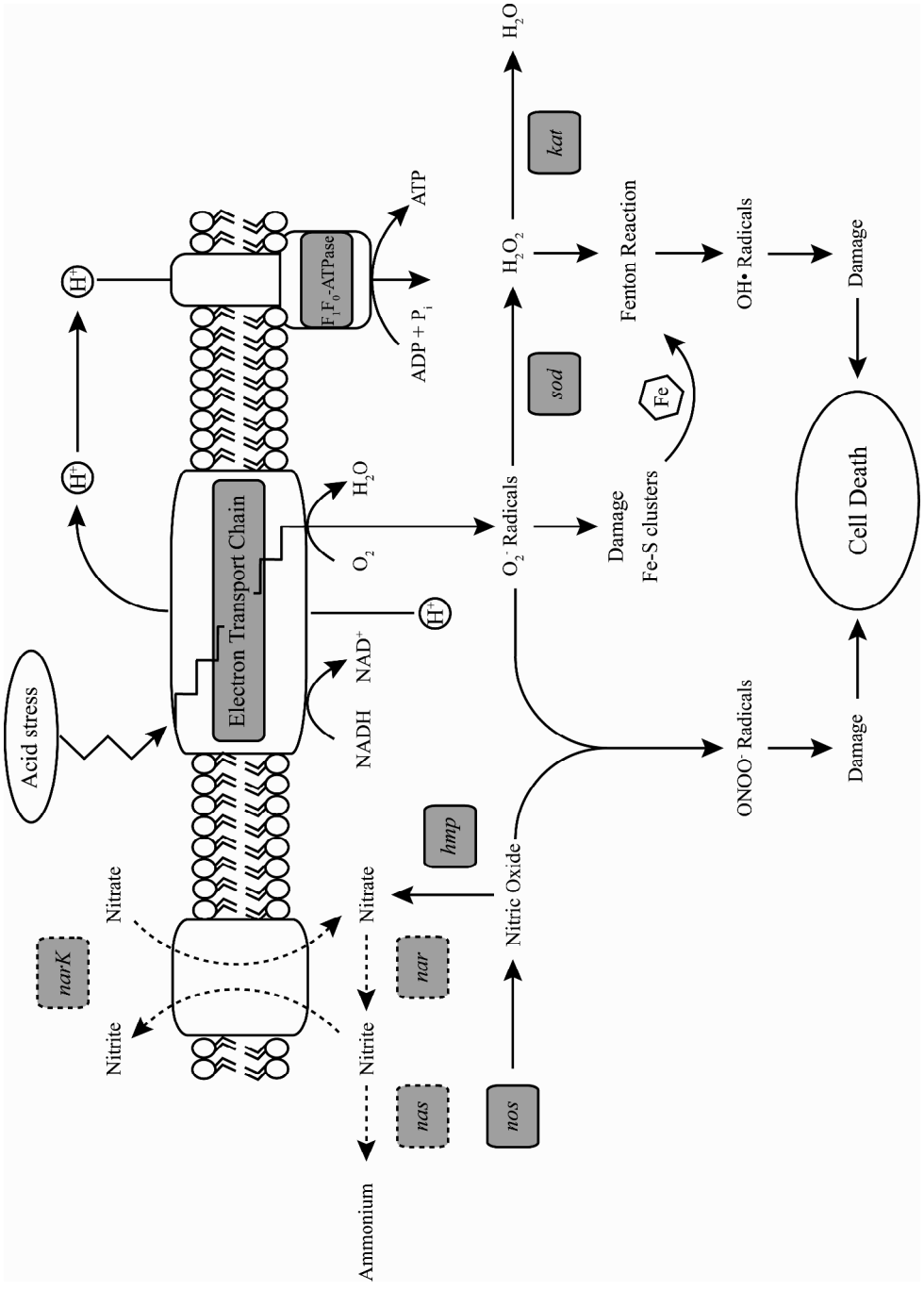
Acid stress
Electron Transport Chain
NADH
NAD+
O2
H2O
H+
F1F0-ATPase
ATP
ADP + Pi
narK
Nitrate
Nitrite
nar
nas
nos
hmp
Nitric Oxide
O2- Radicals
H2O2
H2O
sod
kat
Damage
Fe-S clusters
Fe
Fenton Reaction
OH• Radicals
ONOO- Radicals
Damage
Damage
Cell Death
Ammonium

Fig. 8. Low pH induced oxidative stress response and radical forming mechanisms in *B. cereus* ATCC 14579 and ATCC 10987. Schematic representation of radical formation conceivably induced upon exposure to lethal acid stress. Acid stress may cause perturbation of the electron transfer chain and an excess of superoxide radicals (O_2^-) may be formed. Superoxide radicals can be converted to hydrogen peroxide and water by superoxide dismutase (*sod*) and catalase (*kat*). However, when the capacity to dismutate superoxide is not sufficient, free superoxide radicals can cause damage to iron-sulphur (Fe-S) cluster containing enzymes supplying unbound iron ions. These free iron ions and hydrogen peroxide can react (Fenton reaction) and produce hydroxyl radicals (OH·). Another possible route in forming highly damaging radicals may occur via nitric oxide. Nitric oxide can be formed by nitric oxide synthase (*nos*) and can react with superoxide radicals to form peroxynitrite ($ONOO^-$). Nitric oxide can be converted to nitrate by nitric oxide dioxygenase (*hmp*). Subsequently, nitrate can be converted to nitrite and ammonium by nitrate (*nar*) and nitrite (*nas*) reductase, respectively. Nitrite can also be transported outside the bacterial cell by a Nitrite extrusion protein (*nark*). *nar*, *nas* and *narK* are ATCC 14579 specific, the reactions they catalyze are indicated with dotted lines.

potentially involved in ETC activity. This disturbance may cause premature leakage of electrons to oxygen leading to the formation of superoxide (O_2^-). Indeed, elevated levels of O_2^- could be detected in *B. cereus* cells upon exposure to lethal levels of acidity as indicated by staining of these cells with a superoxide-specific fluorescent probe (Mols *et al.*, unpublished results). Furthermore, the formation of O_2^- can be inferred from the induction of superoxide dismutase and catalase genes. Iron-sulphur clusters may subsequently be damaged by O_2^- releasing iron in the cytoplasm (Imlay, 2006). Free iron can react with hydrogen peroxide, originating from the dismutation of O_2^-, forming the highly toxic OH· radicals in the Fenton reaction (Imlay *et al.*, 1988). Furthermore, O_2^- can rapidly react with nitric oxide (NO) to form another highly toxic oxidative compound, $ONOO^-$ (Beckman and Koppenol, 1996). NO is formed by a reaction catalyzed by nitric oxide synthase (bNOS). Indirect indications for the formation of NO upon low pH exposure can be inferred from the up-regulation of nitric oxide dioxygenase and nitric oxide dependant regulator *dnrN*. The induction of bNOS activity, which is possibly regulated at protein level (Shatalin *et al.*, 2008), may initially have a positive effect on surviving oxidative stress. bNOS-derived NO may inhibit thiol reduction leading to the inhibition of the OH· forming Fenton reaction (Gusarov and Nudler, 2005; Sudhamsu and Crane, 2009). Furthermore, NO induces catalase activity in *B. anthracis* (Shatalin *et al.*, 2008) and inhibits the aerobic ETC (Husain *et al.*, 2008). On the other hand, NO facilitates the formation of $ONOO^-$, which may have a damaging effect that could lead to cell death. Nitric oxide dioxygenase and nitrite reductase are described to be possible NO dissipation routes (Payne *et al.*, 1997; Gardner, 2005). The genome of ATCC 14579 encodes both mechanisms and this strain showed to be more acid resistant than the nitrite/nitrate reductase deficient ATCC 10987 strain.

The phenomenon that exposure to stresses such as salt, heat, acid, and bile, results in secondary oxidative stress, has been described earlier for *B. cereus* and numerous other bacteria (Aldsworth *et al.*, 1999; Clements *et al.*, 1999; Hecker and Volker, 2001; Airo *et al.*, 2004; Banjerdkij *et al.*, 2005; Latifi *et al.*, 2005; Dodd *et al.*, 2007; Kim *et al.*, 2008), but up to now, this secondary oxidative stress response has not been linked to radical-

associated cell death. Moreover, our findings are supported by earlier observations in amongst others *S. aureus* and *Vibrio vulnificus*, where acid resistance was found to be superoxide dismutase (and catalase) dependant (Clements and Foster, 1999; Kim *et al.*, 2005).

In conclusion, the results obtained in our study provide evidence for the origin of acid stress-induced oxidative stress. The relative contribution to inactivation of cells of the radicals involved, i.e., $OH\cdot$ and $ONOO^-$, remains to be elucidated. In extension to the antibiotic study of Kohanski and colleagues (2007), we now propose that in aerobic conditions, the formation of radicals such as $OH\cdot$ and $ONOO^-$ may be a common mechanism of cellular death in bacteria exposed to severe stress conditions.

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Supplementary material

The following supplementary material is available for this chapter online http://www.fhm.wur.nl/uk/thesismaarten: supplementary microarray analyses including tables and figures.

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Chapter 6

The impact of oxygen availability on stress survival and radical formation of *Bacillus cereus*

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Abstract
Both the growth and stress survival of two model *Bacillus cereus* strains, ATCC 14579 and ATCC 10987, were tested in three different conditions varying in oxygen availability, i.e., aerobic, microaerobic and anaerobic conditions. Both *B. cereus* strains displayed highest growth rates and yields under aerobic conditions, whereas the microaerobic and anaerobic cultures showed similar reduced growth performances. The cells grown and exposed microaerobically and anaerobically were more resistant to heat and acid than cells that were cultured and exposed aerobically. On the other hand, the anaerobically grown cells were more sensitive to hydrogen peroxide compared to the (micro)aerobically grown cells. The increased heat- and acid-induced inactivation in aerobic conditions appeared to be associated with intracellular accumulation of excess hydroxyl and/or peroxynitrite radicals, as determined by flow cytometry in combination with the fluorescent reporter dye 3'-(p-hydroxyphenyl) fluorescein. This suggests that radical formation may contribute to inactivation of bacteria in the presence of oxygen, such as in aerobic and microaerobic conditions. No evidence was found for radical formation upon exposure to salt and hydrogen peroxide. The increased resistance to heat and acid in microaerobic and anaerobic conditions shows that oxygen availability should be taken into account when behaviour of bacteria, such as *B. cereus*, in food industry related conditions is investigated, because oxygen availability may affect the efficiency of food preservation conditions.

Introduction

Bacillus cereus is a Gram-positive, ubiquitously found, spore-forming organism, which can be isolated from soil (Vilain *et al.*, 2006) and foods (Kotiranta *et al.*, 2000), such as milk (Larsen and Jorgensen, 1999), rice (Dufrenne *et al.*, 1994) and vegetables (Choma *et al.*, 2000). Furthermore, it is a notorious spoilage organism with a substantial economic impact (Te Giffel, 2001) and it can cause two types of food-borne illnesses, emesis and diarrhoea. The diarrheal type of illness is caused upon formation of several enterotoxins inside the human gastro-intestinal tract (Granum and Lund, 1997; Kotiranta *et al.*, 2000). The emetic type of illness is caused by the formation of cereulide (or emetic toxin) in food that is subsequently ingested (Turnbull, 1981; Agata *et al.*, 2002). Often the symptoms of *B. cereus*-induced illnesses are mild, and therefore food-borne illnesses caused by *B. cereus* are frequently not reported. However, in very rare cases a subsequent systemic infection can be fatal (Dierick *et al.*, 2005).

In food, during food processing and storage, and in other environments *B. cereus* can encounter various adverse conditions, e.g., high temperature, low pH, high salt concentrations and exposure to oxidative compounds. Vegetative cells can be heat-inactivated in for instance pasteurization procedures, and therefore the heat stress response of *B. cereus* has been studied in detail (Browne and Dowds, 2001; Periago *et al.*, 2002; Van Schaik *et al.*, 2004). The response of *B. cereus* to salt stress and acid stress has also been studied (Browne and Dowds, 2001, 2002; Jobin *et al.*, 2002; den Besten *et al.*, 2006; Thomassin *et al.*, 2006), showing that exposure to NaCl can protect *B. cereus* against other subsequent stress exposures (Browne and Dowds, 2001; den Besten *et al.*, 2006) and that *B. cereus* exhibits an acid tolerance response (Jobin *et al.*, 2002). Furthermore, it has been shown that the exposure to stresses influences the expression of virulence factors (Thomassin *et al.*, 2006), which may have an impact on the pathogenicity of this organism. Oxidizing agents, such as hydrogen peroxide, are used to clean food processing equipment (Dunsmore *et al.*, 1981). The response of *B. cereus* to hydrogen peroxide has been investigated by Browne and Dowds (Browne and Dowds, 2001), showing that pre-exposure to hydrogen peroxide stress leads to increased resistance to heat and ethanol.

Although *B. cereus* is a facultative anaerobic organism, earlier studies on stress responses of *B. cereus* were always conducted in the presence of oxygen. Anaerobic growth of *B. cereus* is supported by fermentation and/or anaerobic respiration (Rosenfeld *et al.*, 2005) and may enhance its performance in oxygen-deprived environments, such as soil (Vilain *et al.*, 2006), vacuum-packed foods (Tham *et al.*, 2000), ready-to-eat meals (Rosenquist *et al.*, 2005), the human GI-tract (Laohachai *et al.*, 2003; Stintzi *et al.*, 2005) and insect gut (Brune *et al.*, 2000). Although a number of studies have been conducted on fermentative growth of *B. cereus* (Duport *et al.*, 2004; Rosenfeld *et al.*, 2005; Duport *et al.*, 2006; Zigha *et al.*, 2006, 2007), the need for data on the stress response in oxygen limited conditions is apparent. The fermentation studies have shown that several readjustments occur (Rosenfeld *et al.*, 2005; Zigha *et al.*, 2006), and that *B. cereus* virulence may also be affected by

oxygen limitation, because enterotoxin formation is enhanced in such conditions (Duport *et al.*, 2004; Duport *et al.*, 2006; Zigha *et al.*, 2007).
Recently, it has been shown that the formation of reactive oxygen species plays a role in the inactivation of aerobically grown and exposed cells of *Escherichia coli* and *Staphylococcus aureus* by bactericidal antibiotics (Kohanski *et al.*, 2007). The formation of hydroxyl radicals (OH·) occurred via perturbation of the electron transfer chain producing superoxide. The formation of superoxide can lead to concomitant damage to iron-sulphur clusters releasing iron from these clusters. Iron and hydrogen peroxide, which is formed by the dismutation of superoxide, react producing OH·. Food preservation related stresses such as heat, acid and salt stress may also cause malfunctioning of cellular processes leading to radical formation. However, in anaerobic conditions oxygen is not available for formation of superoxide and in anaerobic growth, alternatives for the aerobic respiration machinery, such as nitrate reductase, may be used. It is conceivable that inactivation effects of food preservation conditions include the formation of radicals, such as OH·, in (micro)aerobic conditions. However, up to now this topic has not been addressed. Therefore in this study, the effect of oxygen limitation on the growth and the heat, acid, salt and hydrogen peroxide resistance of two model *B. cereus* strains, ATCC 14579 (type strain) and ATCC 10987 (isolate from spoiled cheese), was investigated in aerobic, microaerobic and anaerobic conditions. Subsequently, the impact of oxygen limitation on the concomitant formation of radicals in these conditions was assessed.

Materials and methods

Bacterial strains and culture conditions
The *B. cereus* strains ATCC 14579 and ATCC 10987 used in this study were obtained from the American Type Culture Collection (ATCC). Stock cultures grown in brain heart infusion (BHI, Becton Dickinson, France) broth were stored at -80°C in 33% (v/v) glycerol. To prepare aerobic pre-cultures, 20 ml BHI in a 100 ml Erlenmeyer flask was inoculated with a droplet from the glycerol stock. Anaerobic pre-cultures were prepared in 50 ml BHI in a 100 ml air-tight infusion flask, capped with a rubber stopper (Rubber, Hilversum, The Netherlands) and sealed with a metal seal (Omnilabo, The Netherlands), that was flushed for 2 hours after sterilization with filter-sterile nitrogen gas, where after the culture was inoculated as described above. The pre-cultures were incubated overnight at 30°C, shaking at 200 rpm. Aerobic and anaerobic cultures were obtained by inoculating 50 ml BHI in a 250 ml Erlenmeyer flask or in a 100 ml infusion flask, respectively, with 100 µl corresponding pre-culture and incubation at 30°C and shaking at 200 rpm (Rosenfeld *et al.*, 2005). 50 ml Falcon tubes (Greiner Bio-one, Germany) with 50 ml BHI were inoculated with 100 µl aerobic pre-culture and incubated non-shaking at 30°C overnight to allow for microaerobic growth of the strains as previously described (Nakano *et al.*, 1996; Reents *et al.*, 2006).

Growth was monitored by measuring the optical density at 600 nm (OD, Novaspec II, Pharmacia Biotech, Germany). In separate experiments the discoloration of resazurin (0.2 mg/l final concentration, Janssen Chemica, Belgium) was monitored during growth to ensure that the conditions were fully anaerobic (Rosenfeld *et al.*, 2005).
The log OD values, obtained in the growth experiments, were fitted with a modified Gompertz model using TableCurve 2D (Windows v.2.03). The parameters as formulated by Zwietering and colleagues (Zwietering *et al.*, 1990) were modified and should be interpreted as $\log_{10}OD(\infty)$ representing the log final OD, μ (/h) is the specific growth rate in $\log_{10}$ OD per hour, λ (h) represents the initial time to show an increase of $\log_{10}$ OD and $\log_{10}OD(0)$ represents the $\log_{10}$ starting OD (Equation 1). Student's *t* tests (two-sided) were performed in order to compare the average parameter estimates for the different growth conditions and test the statistical significance with $P<0.05$ considered significant.

$$\log_{10}OD(t) = \log_{10}OD(0) + (\log_{10}OD(\infty) - \log_{10}OD(0)) \cdot \exp\left\{-\exp\left[\frac{\mu \cdot e}{(\log_{10}OD(\infty) - \log_{10}OD(0))} \cdot (\lambda - t) + 1\right]\right\} \quad (1)$$

Assessment of stress survival
The heat, acid, salt and hydrogen peroxide stress survival of aerobic, microaerobic and anaerobic cultures at OD ~0.5 was assessed. To expose the various cultures to heat, the different cultures were placed from a water bath at 30°C to a 50°C water bath. To generalize the heat transfer of the three types of cultures, 50 ml of the aerobic and microaerobic cultures were poured into 100 ml infusion flasks (as used for anaerobic cultures) before exposure to 50°C. In a separate experiment, during the exposure to 50°C the temperature of the cultures was monitored with a digital thermometer (TFX 392 SK, Gullimex Instruments, Germany). The cultures reached 50°C (±0.5°C) in approximately 5 minutes, therefore besides the general exposure times of 0, 10, 20, and 30 minutes, a sample was also taken at 5 minutes. To expose the various cultures to pH 4.3, a pre-defined amount of hydrochloric acid (HCl, 37%, Merck, Germany) was added to the 50 ml cultures. The pH was measured after addition of HCl with a PHM 240 pH/ION Meter (Radiometer, Denmark). The cells were exposed to salt stress by first removing 13 ml of the 50 ml cultures. To the remaining 37 ml of the cultures 12 ml of a 25% (w/v) NaCl (VWR, Belgium) solution to obtain a final concentration of 6% (w/v) NaCl. This method allowed for similar salt stress exposures and efficient mixing of the three types of cultures including the microaerobic cultures. The hydrogen peroxide stress resistance of *B. cereus* was assessed by adding 1.67 ml of a 30% (v/v) stock solution of H_2O_2 (Merck, Germany) to the various 50 ml cultures obtaining a final concentration of 1% (v/v) H_2O_2. 1 ml samples were taken at 0, 10, 20 and 30 min after exposure to the different stresses tested, serially diluted in peptone physiological salt solution (PPS, 1 g/l neutralized bacteriological peptone (Oxoid, England) and 8.5 g/l NaCl in water) and the dilutions were plated on BHI agar plates (15 g/l bacteriological agar [Oxoid, England]) and incubated overnight at 30°C under aerobic conditions. The mean values and standard deviations were calculated and the

statistical significant differences were determined using Student's *t*-tests with $P<0.05$ considered significant.

Detecting radical formation using flow cytometry

To detect radical formation in stress-exposed *B. cereus* cells, the fluorescent reporter dye 3'-(p-hydroxyphenyl) fluorescein (HPF, Invitrogen, The Netherlands) was used (Setsukinai *et al.*, 2003). HPF reacts with hydroxyl (OH·) radicals and peroxynitrite ($ONOO^-$) to form green fluorescent fluorescein. At designated time points (0, 10, 20, 30 minutes) after exposure to the tested stresses, samples were obtained by centrifuging (15,000 × *g*, 30 s) 1 ml of culture of the exposed exponentially growing cultures (OD_{600} ~0.5). The cell pellet was resuspended in 1 ml ice cold, filtered (0.2 µm, FP30/0.2 CA-S, Whatman, Germany) phosphate buffered saline (PBS). The PBS used with anaerobic samples was flushed with nitrogen gas prior to use. The samples were washed once and diluted with filtered PBS to obtain a concentration of approximately 10^6 cells per ml. HPF was added to the washed and diluted cells at a final concentration of 5 mM. The data were collected using a Becton Dickinson FACSCalibur flow cytometer with the following photomultiplier tube (PMT) voltage settings: E00 (FSC), 360 (SSC) and 825 (FL1). Data were obtained from 20,000 events (cells) at medium flow rate using Cellquest Pro software (version 4.0.2) and were analyzed with WinMDI 2.9 (Joseph Trotter, Salk Institute for Biological Studies, La Jolla, California, USA; http://facs.scripps.edu/software.html). Overlay figures were constructed with Adobe Illustrator CS2 (version 12.0.1).

Results

Aerobic, microaerobic and anaerobic growth

The aerobic, microaerobic and anaerobic cultures of *B. cereus* strains ATCC 14579 and ATCC 10987 were tested with resazurin to assess the oxygen levels present. In the anaerobic cultures resazurin was decoloured prior to inoculation showing that there were no resazurin-detectable levels of oxygen present (data not shown). The aerobic and microaerobic cultures did show detectable levels of oxygen prior to inoculation. Before reaching OD ~0.5, both aerobic and microaerobic cultures were decoloured, indicating that the growing cells consume most of the oxygen present in the cultures and leaving no resazurin-detectable oxygen levels (data not shown). The influx of oxygen differentiates aerobic and microaerobic cultures, with the cells in the aerobic cultures presumably consuming all oxygen that enters by diffusion instantaneously.

The impact of the three different types of culturing on the growth of *B. cereus* strains ATCC 14579 and ATCC 10987 is shown in Figure 1. The parameter estimates of fitting the modified Gompertz equation (Equation 1) showed that there were no significant differences between the growth of the two strains tested with the different culturing methods, except for the higher specific aerobic growth rate (μ) of strain ATCC 10987 (Table 1). In aerobic conditions, both strains showed a significant greater growth rate and a higher final OD

($\log_{10}$ $OD(\infty)$) compared to growth in the other two conditions. Despite the different oxygen levels at the start between the anaerobic and microaerobic culture conditions, the growth of *B. cereus* was not significantly different between the two conditions. Thus, *B. cereus* grows faster and can reach a higher final OD when there is an influx of oxygen into the cultures.

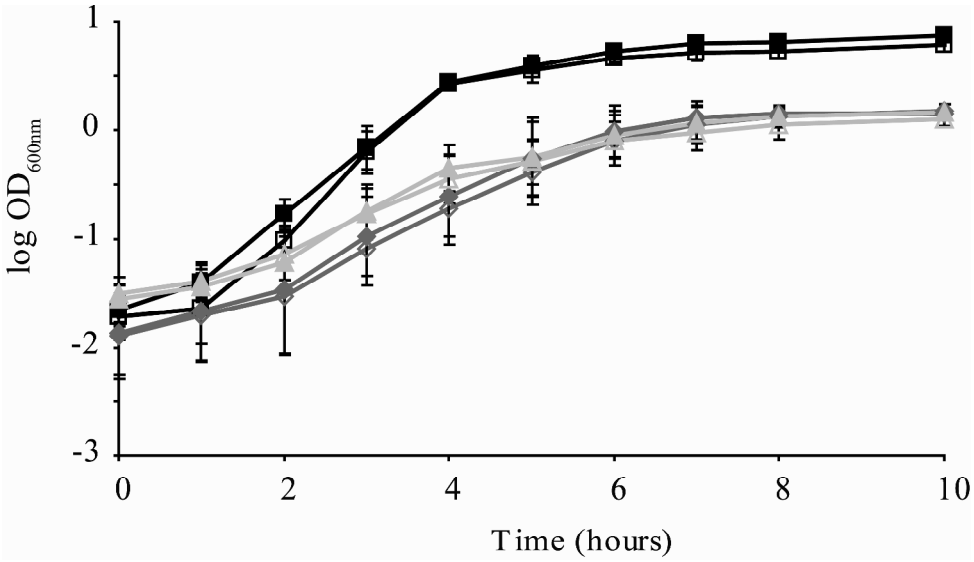

Fig. 1: Effect of oxygen availability on the growth of *B. cereus* strains ATCC 14579 (closed symbols) and 10987 (open symbols). The log OD of the cultures grown aerobically (black lines and squares), microaerobically (dark grey lines and diamonds) and anaerobically (light grey lines and triangles) was measured in time. The error bars represent the standard deviation of the average of triplicate experiments.

Table 1: Parameter estimates of the reparameterized Gompertz model

		Parameter value[a]			
Strain	Growth condition	$\log_{10} OD(\infty)$	μ	λ	$\log_{10} OD(0)$
ATCC 14579	Aerobic	0.86^{x}	0.74^{x}	0.70	-1.73
	Microaerobic	0.19^{y}	0.48^{y}	0.92	-2.04
	Anaerobic	0.21^{y}	0.43^{y}	1.58	-1.52
ATCC 10987	Aerobic	0.74^{x}	0.93^{z}	1.05	-1.89
	Microaerobic	0.27^{y}	0.43^{y}	1.00	-1.94
	Anaerobic	0.09^{y}	0.40^{y}	1.38	-1.49

[a] The values in the columns representing the parameters $\log_{10} OD(\infty)$ and μ (/h) with different letters (^{x}, ^{y}, ^{z}) were significantly different (P<0.05). The $\log_{10} OD(\infty)$ was not significantly different between the strains, and the μ was significantly different (P<0.05) between the strains when grown aerobically. In the columns representing λ (h) and $\log_{10} OD(0)$ no differences were significant.

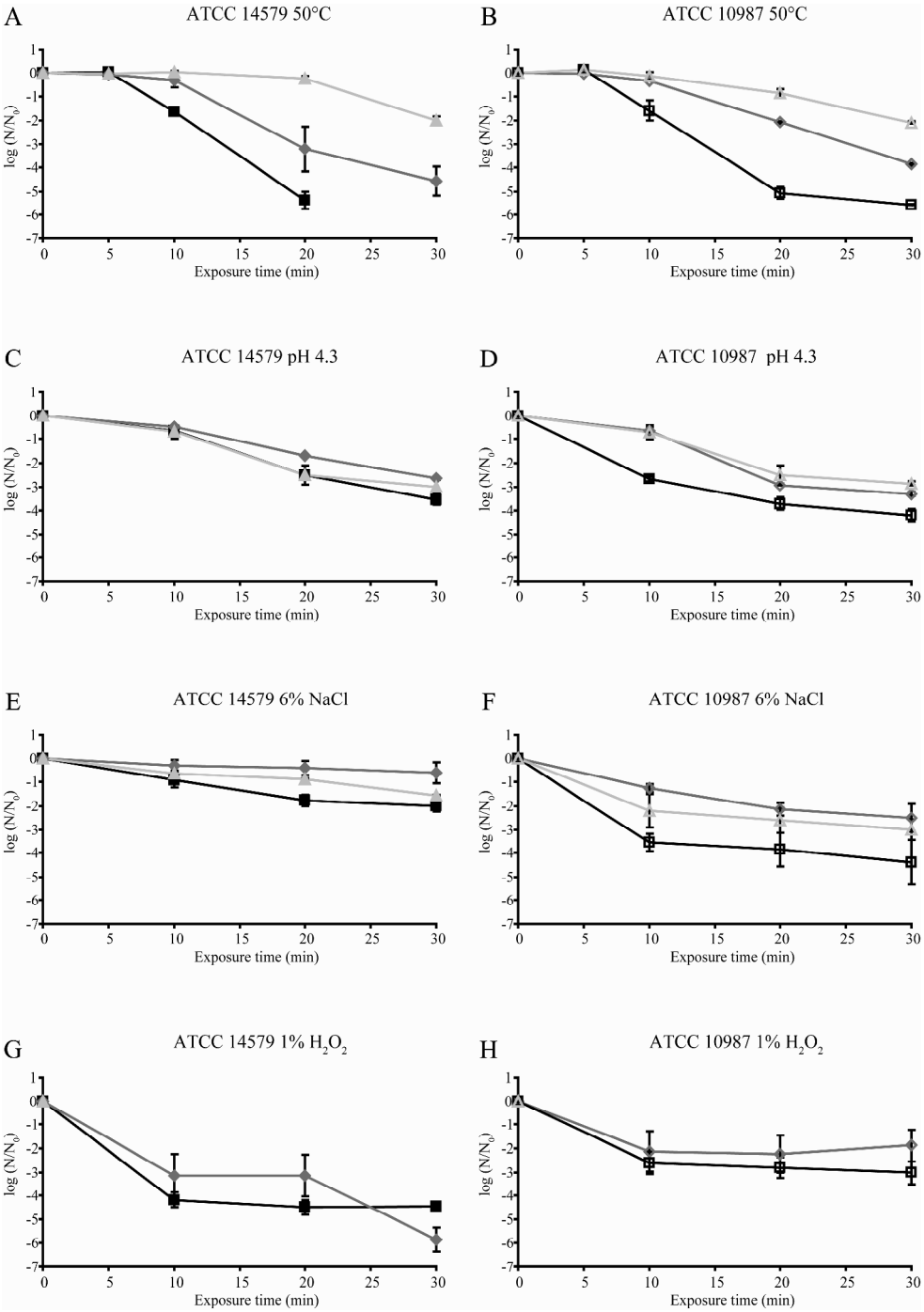
A
ATCC 14579 50°C
B
ATCC 10987 50°C
C
ATCC 14579 pH 4.3
D
ATCC 10987 pH 4.3
E
ATCC 14579 6% NaCl
F
ATCC 10987 6% NaCl
G
ATCC 14579 1% H2O2
H
ATCC 10987 1% H2O2
log (N/N0)
Exposure time (min)

Fig. 2: Effect of oxygen availability on heat, acid, salt and hydrogen peroxide stress survival of *B. cereus* ATCC 14579 (closed symbols, A, C, E and G) and ATCC 10987 (open symbols, B, D, F and H). Aerobically grown and exposed cultures (black lines and squares), cultures grown microaerobically (dark grey lines and diamonds) and anaerobically grown and exposed cultures (light grey lines and triangles) of OD ~0.5 were subjected to 50°C (A and B), pH 4.3 (C and D), 6% NaCl (E and F) and 1% H_2O_2 (G and H). Samples were taken at 0, 5 (heat only), 10, 20 and 30 minutes and the colony forming units per ml were counted and expressed in log (N/N_0). The error bars represent the standard deviation of the average.

Impact of oxygen limitation on heat, acid, salt and hydrogen peroxide stress survival

Upon exposure to heat, i.e., 50°C (Fig. 2A and 2B), ATCC 14579 and ATCC 10987 cells grown anaerobically were significantly more resistant than cells grown microaerobically and aerobically. Microaerobically grown cells of both strains were significantly more resistant than aerobically grown cells (Fig. 3A and 3B). There was no significant difference in heat resistance between the two tested strains.

Upon exposure to acid, i.e., pH 4.3 (Fig. 2C and 2D), strain ATCC 10987 showed to be significantly less acid resistant when grown aerobically, compared to the other culturing conditions (Fig. 3B). Also strain ATCC 14579 showed to be significantly less acid resistant when grown aerobically, compared to the microaerobic and anaerobic culturing (Fig. 3A). There was no significant difference between acid sensitivity of microaerobic and anaerobic cells of the two strains. Strain ATCC 10987 appeared to be more sensitive to pH 4.3 than strain ATCC 14579.

Upon exposure to salt, i.e., 6% NaCl (Fig. 2E and 2F), the type strain ATCC 14579 was significantly more resistant than the cheese spoilage strain ATCC 10987 (Fig. 3A and 3B). Both strains were the least resistant to salt when grown aerobically and the most resistant when grown microaerobically, however, the differences observed were not significant (Fig. 3A and 3B).

Upon exposure to hydrogen peroxide, i.e., 1% H_2O_2 (Fig. 2G and 2H), the anaerobically grown and exposed cultures of both strains were rapidly inactivated with the viable counts falling below the detection limit within the first 10 min of exposure. Aerobically and microaerobically grown cells were significantly more resistant to hydrogen peroxide stress than anaerobically grown cells. However, after 30 min of exposure to H_2O_2 the aerobically and microaerobically grown ATCC 14579 cultures were inactivated to such extent that it was not significantly different from the anaerobic culture anymore (Fig. 3A and 3B). ATCC 14579 showed to be more sensitive to hydrogen peroxide than ATCC 10987, with the (micro)aerobically grown cells of the latter strain being significantly more resistant to hydrogen peroxide stress than the anaerobically grown cells.

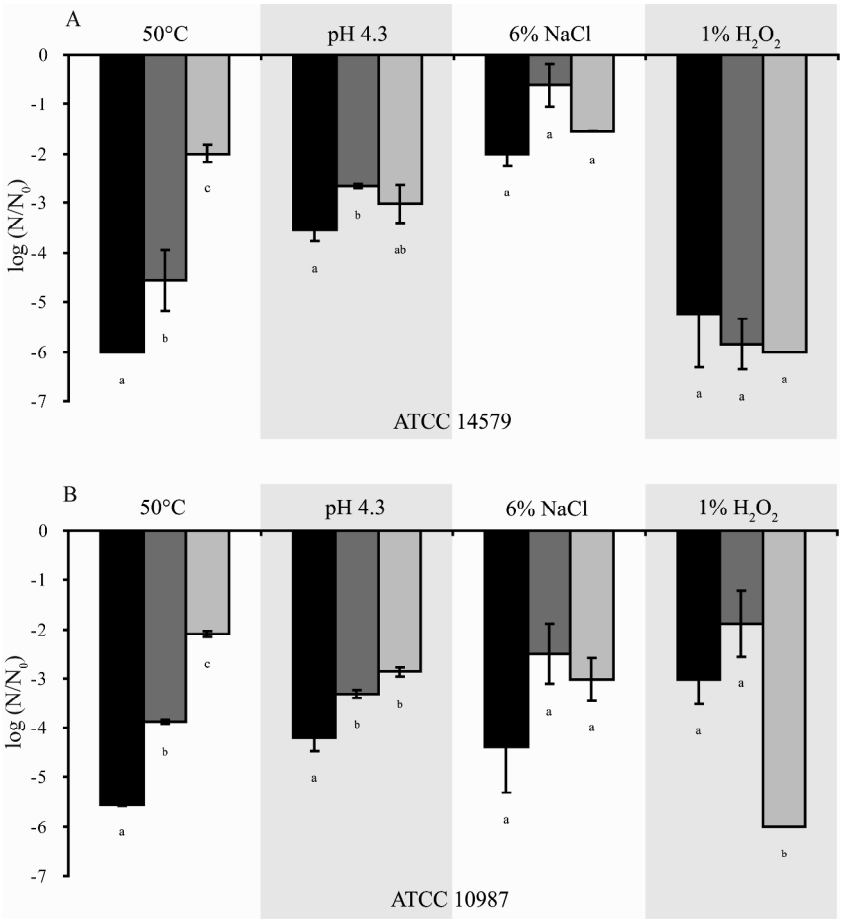

Fig. 3: Effect of oxygen availability on heat, acid, salt and hydrogen peroxide stress survival of *B. cereus* ATCC 14579 (A) and ATCC 10987 (B). Aerobically grown and exposed cultures (black bars), cultures grown microaerobically (dark grey bars) and anaerobically grown and exposed cultures (light grey bars) of OD ~0.5 were subjected to 50°C, pH 4.3, 6% NaCl and 1% H_2O_2 as indicated at the top. Samples were taken at 0 and 30 minutes and the colony forming units per ml were counted and expressed in log (N/N_0). Error bars represent the standard deviation of the average at the corresponding point, significant differences (P<0.05) between the culturing conditions are indicated with different letters (a, b, c).

Radical formation upon stress exposure

The formation of $OH\cdot$ and $ONOO^-$ was investigated by flow cytometry analysis of cells stained with the fluorescent probe HPF. The formation of excess radicals could be observed, as shown by an increased fluorescence (Fig. 4, Fig. 5 and Fig. 6). Histogram plots obtained of aerobically grown *B. cereus* ATCC 14579 and ATCC 10987 exposed to

heat (A), acid (B), salt (C) and hydrogen peroxide (D) are shown in Fig. 4 and Fig. 5, respectively. Both strains showed a clear increase in fluorescence, when the cells were exposed to 50°C and pH 4.3, indicating that an excess of radicals was formed upon exposure to heat and acid. H_2O_2 induced an insignificant small shift in fluorescence and no shift was apparent upon exposure to salt.

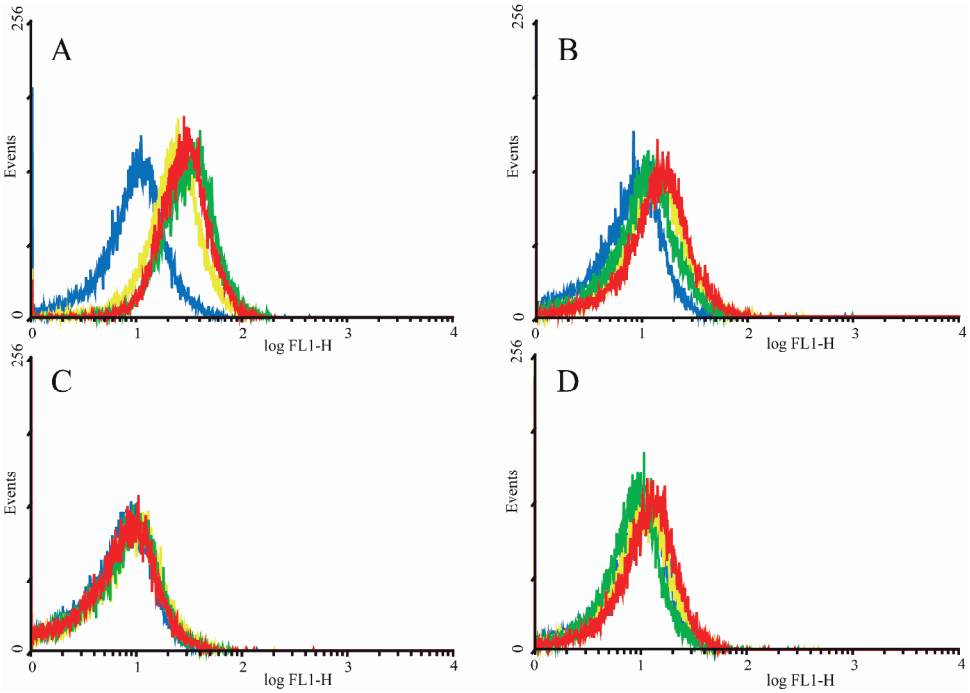

Fig. 4: Radical formation in *B. cereus* ATCC 14579 upon exposure to heat, acid, salt and hydrogen peroxide. The cultures were grown aerobically to OD ~0.5 and exposed to 50°C (A), pH 4.3 (B), 6% NaCl and 1% H_2O_2 (D). Samples were taken from the challenged cultures at 0 (blue), 10 (green), 20 (yellow) and 30 (red) minutes. Representative figures are shown, where the fluorescent signal is plotted against the number of cells. An increase of fluorescent signal (shift to the right) corresponds with the formation of radicals.

Formation of OH· and $ONOO^-$ depends on the presence of superoxide (O_2^-) (Imlay *et al.*, 1988; Pryor and Squadrito, 1995; Beckman and Koppenol, 1996). Superoxide can be formed via pre-mature leakage of electrons to oxygen in the electron transfer chain. Therefore, a role of oxygen in the formation of reactive oxygen species, such as OH· and $ONOO^-$ was expected. The formation of OH· and $ONOO^-$ was measured in cells grown aerobically, microaerobically and anaerobically, after exposure to heat (50°C), because this stress rendered the largest shift in fluorescence (Fig. 5). Aerobically grown cultures of

ATCC 14579 and ATCC 10987 showed an increase in fluorescence, indicating the formation of excess radicals. Cells grown microaerobically also displayed a shift in fluorescence, although less pronounced than that of cells grown aerobically. Anaerobically grown cells exposed to heat did not display excess radical formation. In conclusion, the amount of highly toxic radicals, such as OH· and $ONOO^-$, formed by *B. cereus* upon exposure to heat stress clearly depends on the availability of oxygen with no radicals formed in anaerobic conditions.

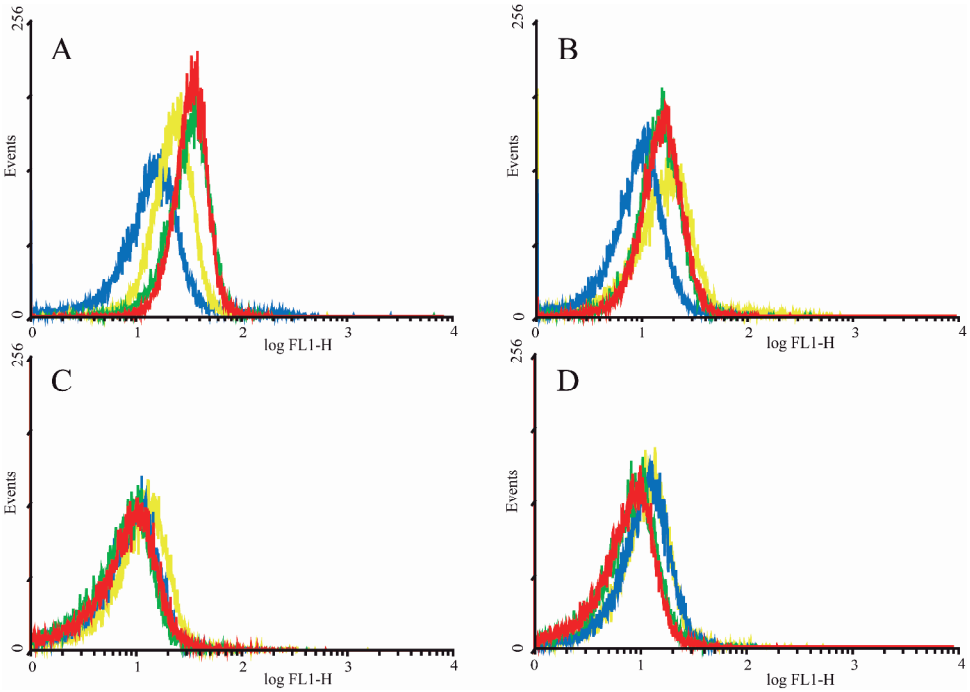

Fig. 5: Radical formation in *B. cereus* ATCC 10987 upon exposure to heat, acid, salt and hydrogen peroxide. The cultures were grown aerobically to OD ~0.5 and exposed to 50°C (A), pH 4.3 (B), 6% NaCl and 1% H_2O_2 (D). Samples were taken from the challenged cultures at 0 (blue), 10 (green), 20 (yellow) and 30 (red) minutes. Representative figures are shown, where the fluorescent signal is plotted against the number of cells. An increase of fluorescent signal (shift to the right) corresponds with the formation of radicals.

Discussion

In this study, the influence of oxygen availability on growth and on survival of *B. cereus* strains ATCC 14579 and ATCC 10987 upon exposure to various food-relevant stresses was investigated. The absence of oxygen, during anaerobic incubation, affected the growth of both strains, resulting in a lower growth rate and a lower yield, which has also been shown

for *B. cereus* F4430/73 (Rosenfeld *et al.*, 2005). Microaerobic culturing is often described as anaerobic, although the medium is not flushed with for example nitrogen gas, to remove oxygen (Nakano *et al.*, 1996; Reents *et al.*, 2006). The growth of *B. cereus* in microaerobic conditions was shown not to be significantly different from anaerobic culturing. This is conceivably due to the rapid depletion of oxygen by the metabolism of the bacteria and the absence of oxygen influx during growth in completely filled and closed culture tubes. Although, *B. cereus* did show similar growth in microaerobic and anaerobic conditions, the response to the various stresses in these conditions was different. Despite the fact that in many environments, where *B. cereus* can be isolated from, oxygen availability is low, most stress response research is conducted on cells grown aerobically. The aerobic response of *B. cereus* to for example heat (Periago *et al.*, 2002), salt (den Besten *et al.*, 2006), acid (Jobin *et al.*, 2002) and bile salts (Kristoffersen *et al.*, 2007) has been investigated. Our study indicates that the influence of oxygen availability on the stress resistance is an important parameter, because *B. cereus* was more resistant to heat and acid, and more sensitive to hydrogen peroxide when grown and exposed in anaerobic conditions. Microaerobic conditions led to inactivation kinetics in between the inactivation kinetics of aerobic and anaerobic conditions. This together with the anaerobic and microaerobic environments *B. cereus* encounters in for instance food industry, i.e., modified atmosphere packaging and fully filled pipelines, makes studying these conditions a necessity for understanding microbial behaviour in food industry relevant conditions.

Aldsworth and colleagues (Aldsworth *et al.*, 1999) proposed that bacteria inactivate themselves upon exposure to stress, the so-called suicide response. This response is caused by the growth arrest induced by relatively mild stresses in actively respiring cells, while their metabolism continues, resulting in an oxidative burst of free radicals. A similar response has been described for *Escherichia coli* and *Staphylococcus aureus* upon exposure to bactericidal antibiotics (Kohanski *et al.*, 2007). Our study showed that the exposure of *B. cereus* strains ATCC 14579 and ATCC 10987 to heat and acid led to the formation of excess radicals, i.e., hydroxyl and/or peroxynitrite. Exposure to 6% salt did not generate detectable radical levels, which indicates that salt did not induce a clear suicide response. Also hydrogen peroxide did not induce a clear shift in fluorescence in *B. cereus* strains ATCC 14579 and ATCC 10987, indicating that detectable levels of $OH\cdot$ and/or $ONOO^-$ are not reached, although, H_2O_2 itself is an oxidative agent that may produce reactive oxygen species. Furthermore, we showed that in *B. cereus* ATCC 14579 and ATCC 10987 cells the formation of excess radicals was dependant on the availability of oxygen, i.e., the increase in fluorescence was only noted in (micro)aerobic conditions, and not with cells grown and exposed in anaerobic conditions.

Anaerobic and microaerobic conditions cause *B. cereus* to grow slower and to form less biomass compared to aerobic growth. Despite these negative effects of oxygen deprivation and/or limitation on growth, the cells became more resistant to heat and acid when grown under these conditions. The increased inactivation of aerobically grown *B. cereus* upon exposure to lethal pH and temperature corresponded with the excess formation of hydroxyl

radicals and/or peroxynitrite. The increased resistance to heat and acid in microaerobic and anaerobic conditions shows that oxygen availability should be taken into account when behaviour of bacteria, such as *B. cereus*, in food industry related conditions is investigated since this may affect the efficiency of food preservation conditions.

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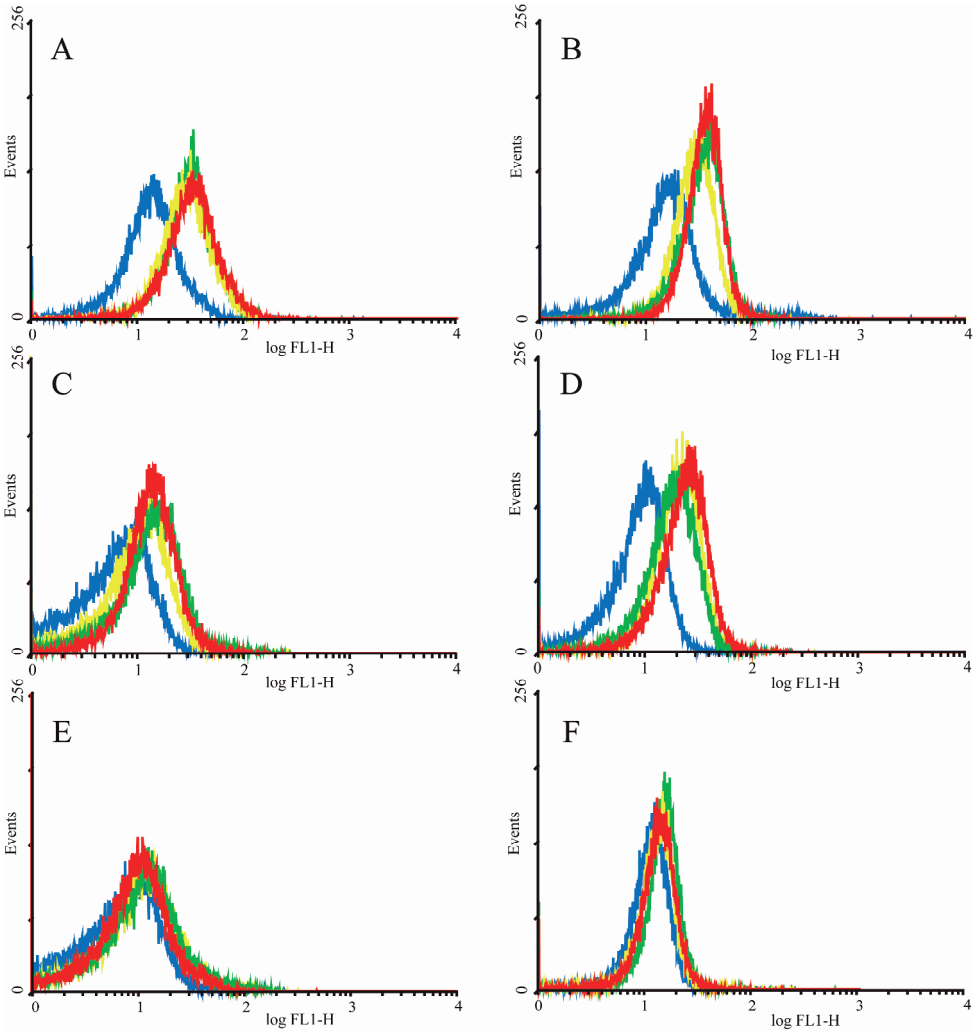

Fig. 6: Effect of oxygen availability on radical formation in *B. cereus* ATCC 14579 and ATCC 10987 upon exposure to heat. Aerobically (A and B), microaerobically (C and D) and anaerobically (E and F) grown and exposed ATCC 14579 (A, C and E) and ATCC 10987 (B, D and F) cells were exposed to 50°C upon reaching OD ~0.5. Samples were taken from the challenged cultures at 0 (blue), 10 (green), 20 (yellow) and 30 (red) minutes. Representative figures are shown, where the fluorescent signal is plotted against the number of cells. An increase of fluorescent signal (shift to the right) corresponds with the formation of radicals.

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Chapter 7

Stress-induced superoxide formation in *Bacillus cereus* detected using the fluorescent probe MitoSOX

Maarten Mols, Mara Ceragioli, and Tjakko Abee
Submitted for publication

Abstract
The red fluorescent probe MitoSOX allows for detection of superoxide, a well-known reactive oxygen species, in bacteria. Single cell analysis using flow cytometry revealed superoxide formation in *Bacillus cereus* cells exposed to a range of stresses, including heat, acid and the oxidative agent tellurite.

Introduction

The role of reactive oxygen species (ROS) in bacterial injury and stress response is becoming more apparent (Dodd *et al.*, 2007; Kohanski *et al.*, 2007; Wright, 2007; Wesche *et al.*, 2009). ROS are inevitably produced in organisms that use oxygen as an electron acceptor and have, also in microorganisms, important biological significance. A goal of current research is to identify the sources of these ROS and to reveal the biomolecules that they damage (Imlay, 2008). One of the ROS, presumably produced upon the premature leakage of electrons to oxygen, is superoxide (O_2^-). Recently, indications were found that ROS, such as hydroxyl radicals and peroxynitrite that are possibly derived from O_2^-, play a role in the response of bacteria to bactericidal antibiotics and to other environmental stresses, including high temperature and low pH (Kohanski *et al.*, 2007; Mols *et al.*, unpublished results). However, a correlation between the formation of hydroxyl radicals and peroxynitrite and the presence of O_2^- has not been established in these conditions.

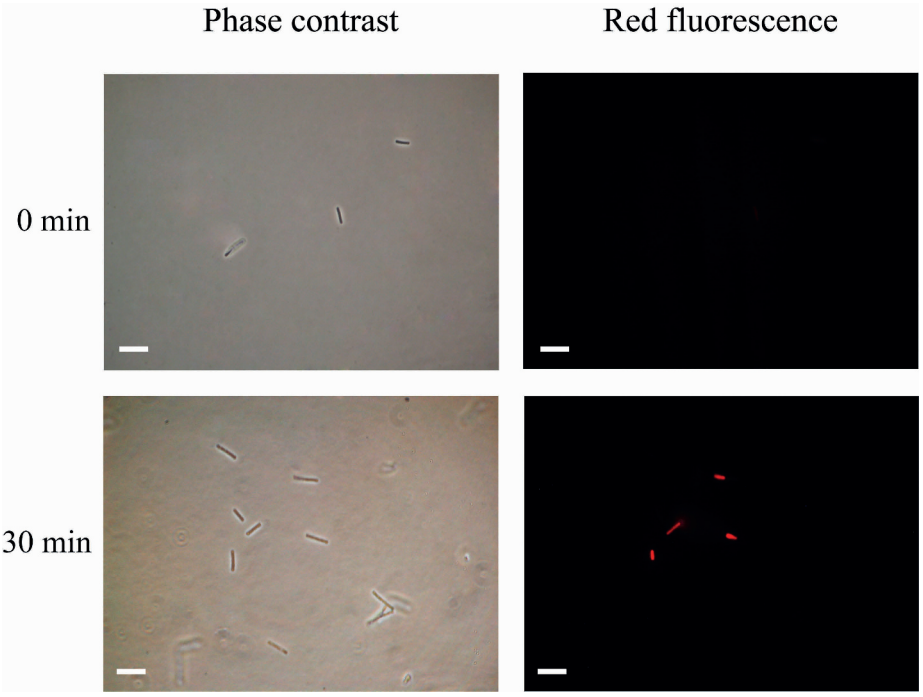

Fig. 1. Phase contrast and fluorescence microscopy images of MitoSOX-stained control and heat-exposed *B. cereus* cells. Cells were exposed to 50°C for 0 (upper row) and 30 min (lower row) and subsequently stained with MitoSOX. Images were obtained using phase contrast (left column) and red fluorescence (right column), showing fluorescence produced by MitoSOX indicative of O_2^- formation. Each image contains a scale bar representing 10 µm.

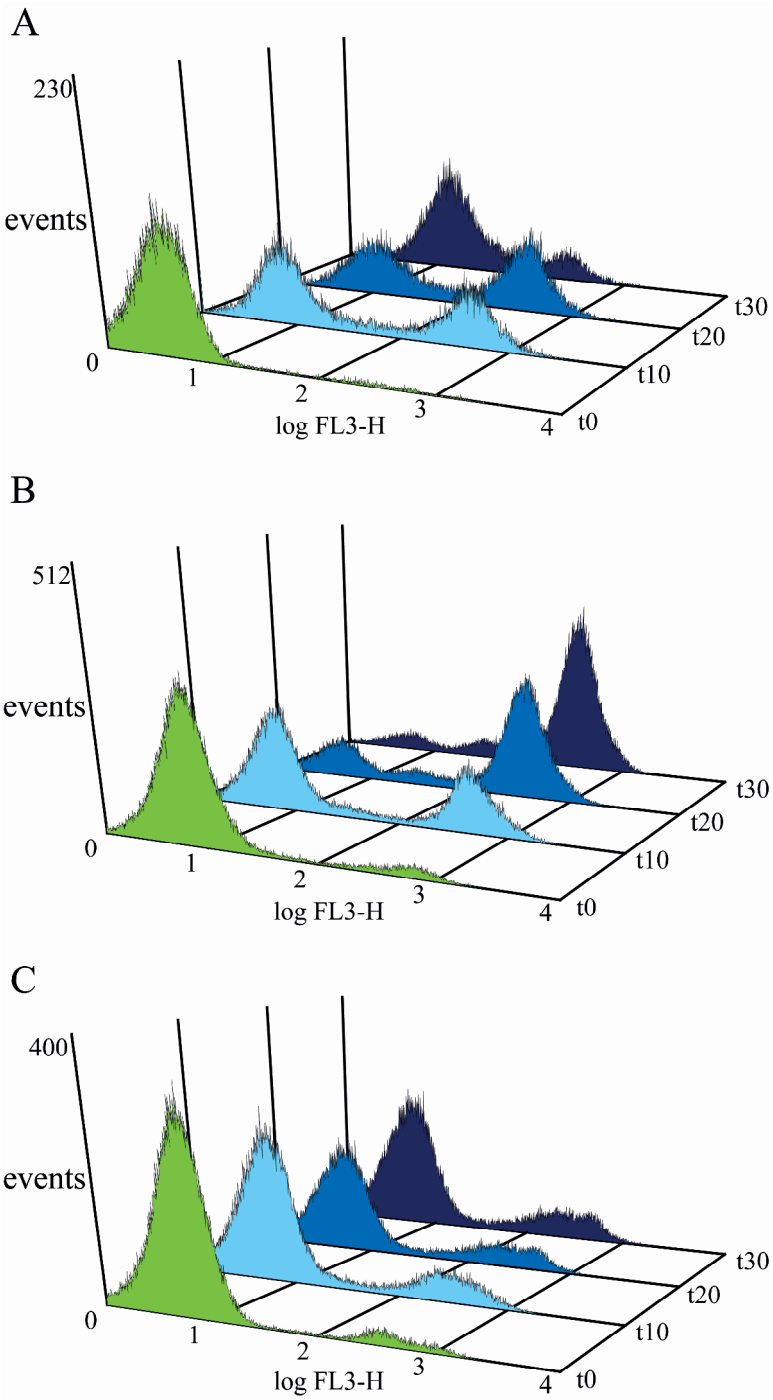
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Fig. 2. Flow cytometry analysis of O_2^--dependant MitoSOX fluorescence in *B. cereus* ATCC 14579 exposed to 50°C (A), pH 4.5 (B), and 1 mg ml^{-1} K_2TeO_3 (C). The red fluorescent signal (log FL3-H) is depicted against the number of events (cells) with a corresponding fluorescence value. Fluorescence values were obtained with unexposed cells (t0, green) and with cells exposed to the different stresses for 10 (light blue), 20 (blue), and 30 min (dark blue). Fluorescence values (log FL3-H) from 2 to 3 comprise the highly red-fluorescent MitoSOX stained cells.

Therefore, we designed a method enabling the detection of O_2^- in bacteria using the hydroethidine-derived fluorescent probe, MitoSOX (Molecular Probes, Invitrogen, USA). The probe is specifically oxidized by O_2^- and subsequently binds DNA to become highly red-fluorescent. Up to now, MitoSOX has only been used for life cell imaging of eukaryotes, where MitoSOX is targeted to the mitochondria, to detect O_2^- resulting from mitochondrial dysfunction (Robinson *et al.*, 2006; Robinson *et al.*, 2008).

Materials and methods

Fluorescence microscopy

To visualize O_2^- formation upon stress exposure in *Bacillus cereus*, exponentially growing cells (approximately 10^7 cells per ml) in brain heart infusion broth (Becton Dickinson, France) were exposed to heat (50°C) for 30 minutes, resulting in inactivation of the cells (more than 5 log decrease) as revealed by plate counts (data not shown). The heat treated cells were spun down (15,000 × *g*, 30 s) and resuspended in cold, filtered (0.2 µm, Eppendorf, Germany) phosphate buffered saline (PBS), and MitoSOX (pre-treated according to manufacturer's instructions) was added to the stressed cells (5 µM final concentration). After incubation at 30°C for 15 minutes, the cells were pelleted and resuspended in cold, filtered PBS. Fluorescence staining of the cells was analyzed using a Zeiss Axioskop fluorescence microscope with a fluorescein isothiocyanate filter set (magnification, ×1,000; Carl Zeiss, Germany). Images were obtained with a Canon Powershot G3 digital camera.

Flow cytometry

Population heterogeneity in stress-induced O_2^- generation was assessed using MitoSOX in combination with flow cytometry (FCM), a high throughput technique allowing single cell analysis and sorting of subpopulations (Veal *et al.*, 2000; Bunthof and Abee, 2002; Czechowska *et al.*, 2008). Exponentially growing *B. cereus* cells were exposed to a range of stresses, including heat (50°C), acid (pH 4.5), and the ROS-generating compound potassium tellurite (K_2TeO_3, 1 mg ml^{-1} final concentration), for 0, 10, 20, and 30 minutes, and stained as described above. The stained cells were diluted to approximately 10^6 cells per ml in cold, filtered PBS. Data were collected immediately after diluting the labelled cells using a FACSCalibur flow cytometer (Becton Dickinson) with photomultiplier tube (PMT) voltage settings: E00 (FSC), 350 (SSC) and 650 (FL3). Data were obtained from varying numbers of events using Cellquest Pro software (version 4.0.2), analyzed with

WinMDI 2.9 (http://facs.scripps.edu/software.html) and graphically presented using Adobe Illustrator CS2 (version 12.0.1).

Results

Superoxide detection using fluorescence microscopy

The analysis of non-stressed and heat-inactivated *B. cereus* cells stained with MitoSOX showed that highly red fluorescent cells were only present in the latter condition, indicating that O_2^- was formed upon heat exposure (Fig. 1).

Population heterogeneity in stress induced superoxide formation

Non-stressed cells showed a low level of fluorescence and upon exposure to heat a population of highly fluorescent *B. cereus* cells appeared (Fig. 2A), pointing to efficient detection of the different (sub)populations with FCM. Conceivably, in the remaining low fluorescent population the levels of O_2^- did not reach or lost threshold levels for detection with MitoSOX. *B. cereus* cells exposed to acid shock also showed the appearance of a large highly fluorescent population (Fig. 2B). Apparently, exposure to both heat and acid induces a secondary oxidative stress associated with the generation of O_2^-, which is in accordance with the superoxide dismutase gene expression found in these conditions (Mols *et al.*, unpublished results). Exposure to inactivating concentrations of ROS generator tellurite also allowed for detection of a highly fluorescent population that was somewhat less pronounced than in heat- and acid-stressed cells (Fig. 2C). Finally, O_2^- formation was indeed found to be dependant on the presence of oxygen, because highly fluorescent cells were not detected in FCM analysis of heat-inactivated anaerobically grown *B. cereus* cells (data not shown).

Superoxide formation in model organisms

MitoSOX-assisted detection of O_2^- was also evaluated in aerobically grown Gram-positive and Gram-negative model organisms. *Escherichia coli* K12 and *Salmonella* Typhimurium LT2 were exposed to 50°C and *Bacillus subtilis* 168 and *Listeria monocytogenes* EGD-e were exposed to 55°C for 30 minutes and all showed highly fluorescent MitoSOX-stained cells (Fig. 3). This indicates that also in these bacterial species the induction of secondary oxidative stress, i.e., formation of O_2^-, is associated with inactivation by heat.

Discussion

Our results show that MitoSOX can be used to detect the formation of O_2^- in Gram-positive and Gram-negative bacteria. In addition, exposure of *B. cereus* to a range of inactivating stresses, including heating, acid shock, and exposure to tellurite, was found to be associated

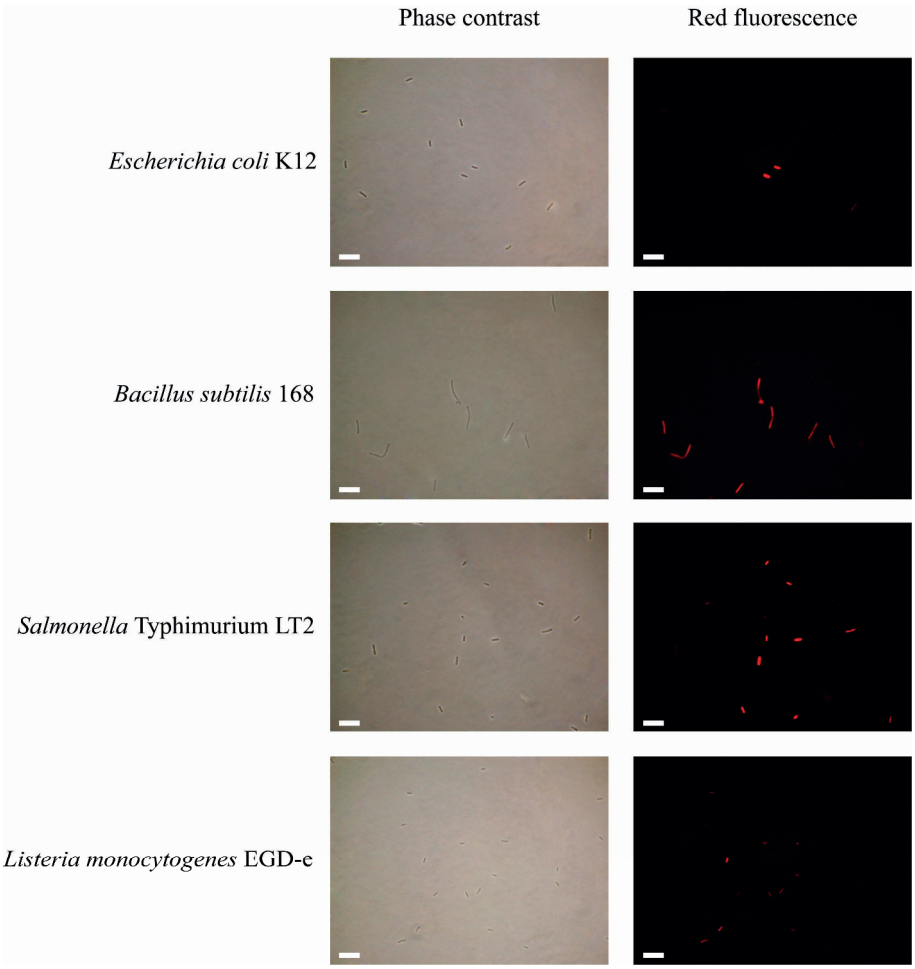

Fig. 3. Phase contrast and fluorescence microscopy images of MitoSOX-stained heat-exposed *E. coli* K12, *B. subtilis* 168, *S.* Typhimurium LT2, and *L. monocytogenes* EGD-e cells forming O_2^-. *E. coli* and *S.* Typhimurium were exposed to 50°C for 30 min and *B. subtilis* and *L. monocytogenes* were exposed to 55°C for 30 min. Subsequently, the cells were stained with MitoSOX. Images were obtained using phase contrast (left column) and a red filter (right column), showing fluorescence signals produced by MitoSOX. Each image contains a scale bar representing 10 µm.

with accumulation of O_2^- in the cytoplasm. Confocal microscopy of red-fluorescent MitoSOX-stained cells revealed a cytoplasmic location of the probe (data not shown), which is in accordance with its proposed fluorescence activation that includes binding to DNA (Robinson *et al.*, 2008). The current protocol does not provide a strict correlation between MitoSOX staining and aerobic cell death. Such a correlation, if existing, requires further studies including refinement of the staining protocol. The use of MitoSOX will allow for further studies on the role of ROS in bacterial stress responses and the identification of corresponding adaptation and survival strategies. The combination with FCM warrants an efficient high throughput analysis enabling assessment of population heterogeneity in O_2^- formation and assessment of its impact on other relevant biological parameters.

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Chapter 8

General discussion and concluding remarks

Introduction

Nowadays, food industries use mild preservation and processing techniques, because consumers demand fresher, healthier and tastier foods and because mild preservation techniques save energy and are more environmental friendly. These mild preservation techniques, such as hurdle technology (Leistner, 2000), may lead to a possible threat: the survival of spoilage and/or pathogenic microorganisms (Abee and Wouters, 1999). For food industries, spoilage and food-borne diseases can have a large economic impact (te Giffel, 2001) and obviously food-borne diseases have a substantial impact on society. Therefore, the adaptive stress response and the physiology of bacteria is an important subject to study.

One of the organisms that can spoil food and cause food-borne illnesses is *Bacillus cereus*. Spores and vegetative cells of *B. cereus* can be found in numerous environments and easily end up in the food chain (Chapter 1). The spores are ultimate survival capsules that can withstand very harsh conditions and are not easy to eradicate in food and food processing equipment (de Vries, 2006). Furthermore, the vegetative cells, that cause spoilage and may produce toxins, are also often exposed to adverse conditions evoking adaptive stress responses that promote survival. To control *B. cereus* in food, knowledge is needed on its physiology and stress response. Within the Food Safety and Preservation (C009) project of Top Institute Food and Nutrition (formerly known as Wageningen Centre for Food Sciences) the demand for such knowledge was addressed. Different survival strategies displayed by *B. cereus*, i.e., spore formation (de Vries, 2006; Hornstra, 2007), biofilm growth (Wijman *et al.*, 2007), and stress response (van Schaik, 2005; den Besten *et al.*, 2006; de Been *et al.*, 2008; van der Voort, 2008), were studied. The stress response research was focussed on investigating underlying mechanisms, amongst others by elucidating the role of transcriptional regulators.

This thesis describes the responses of *B. cereus* upon exposure to low pH environments. *B. cereus* can encounter acid conditions in foods, that often have a low pH with or without the addition of organic acids, and upon ingestion *B. cereus* has to overcome the acid barrier of the human stomach. The acid stress response of Gram-positive organisms, mainly lactic acid bacteria, has been studied. However, the information on the responses displayed by *B. cereus* upon exposure to acid conditions was limited. Some data on the so-called acid tolerance response were obtained, which showed that *B. cereus*' acid stress resistance was increased when the cells were pre-exposed to a mild acidic pH and included modulation of pH_i and protein synthesis (Browne and Dowds, 2002; Thomassin *et al.*, 2006).

The aim of this thesis was to provide mechanistic understanding of acid stress responses of *B. cereus*, because the corresponding survival mechanisms determine its success in a wide range of environments including foods. For the control of *B. cereus* in foods it is especially relevant to understand how *B. cereus* grows and survives in acid conditions. This knowledge may then be applied to optimize food production processes and storage conditions, with the aim of minimizing the threat of *B. cereus*-induced food-borne illnesses.

B. cereus' niche

B. cereus is generally regarded as a soil-dwelling organism that can occupy many niches (Jensen *et al.*, 2003; Stenfors Arnesen *et al.*, 2008). The genomes of strains belonging to the *B. cereus* group showed low numbers of polysaccharide utilization genes, whereas an expanded capacity for amino acid and peptide usage was noted. This led to the suggestion that the common ancestor of the *B. cereus* group co-existed in animal and/or insect hosts (Ivanova *et al.*, 2003; Read *et al.*, 2003). Notably, these assumptions were made based on sequence information of a limited number of strains. In Chapter 2 it was revealed that *B. cereus* ATCC 14579 has the capacity to utilize more carbon sources than initially believed based on genome information alone. ATCC 10987 also acquired other carbohydrate utilization clusters, such as for xylose and tagatose. Nevertheless, it was not able to grow on tagatose, possible due to a lack of tagatose kinase. Furthermore, a discrepancy between suggested physiological capacity based on sequence information and the actual phenotype was also found for the role of urease in *B. cereus* ATCC 10987 (Chapter 3). Rasko and colleagues (2004), assumed a role for ureolytic activity in acid stress resistance when they identified the urease cluster. However, for ten urease positive strains, including ATCC10987, such a role could not be established and ureolytic activity was solely used for growth on urea, i.e., as a source of nitrogen, in eight of these strains. These findings indicate that genome sequences are important tools for understanding behaviour and performances of bacteria, however solid conclusions can only be drawn with support of experimental data.

Species belonging to the *B. cereus* sensu lato group, i.e., *B. cereus*, *Bacillus anthracis*, *Bacillus thuringiensis*, are different species based on their phenotypic characteristics. *B. anthracis* strains have a capsule, and produce a tri-partite toxin. *B. thuringiensis* is recognised by having toxin crystals inside the spores. These characteristics, as well as the capability of making emetic toxin by *B. cereus*, are encoded by genes located on large plasmids and not on the chromosome. Based on the sequences of shared genes, *B. cereus*, *B. anthracis* and *B. thuringiensis* are considered to be one species (Helgason *et al.*, 2000). However, the genomes of the two strains compared in Chapter 2 show large differences. Approximately 80% of the genes on the chromosome are shared and the homology of these genes is close to 100% (Ash *et al.*, 1991). However, close to one fifth of the genes is strain-specific. This diversity within *B. cereus* strains is just as large compared to the differences between *B. cereus*, *B. thuringiensis* and *B. anthracis* without taking the large plasmids into account. Therefore, *B. cereus*, *B. thuringiensis* and *B. anthracis* can be considered one extremely diverse species. Thus, to gain more comprehensive knowledge on behaviour of *B. cereus* as a species, two sequenced strains from different lineages (Chapter 1) were investigated throughout this study.

Much research on Gram-positives organisms is performed on the model organism *B. subtilis*. This species is a generally non-pathogenic soil bacterium that can cause food spoilage and is used to ferment foods, such as natto. The main advantage of studying bacterial behaviour with *B. subtilis* is the fact that it is genetically accessible and naturally

competent. Although related to *B. cereus*, there are many differences between the two species. For instance, the genome of *B. cereus* is generally much larger. *B. subtilis* 168 harbours 4106 orfs and the genome of the *B. cereus* type strain ATCC 14579 encompasses 5255 orfs (ERGO; http://ergo.integratedgenomics.com/ERGO/). It has been suggested that the larger genome of *B. cereus* group members supplies the basis for the occupation of a large diversity of environments. In addition, transcriptional regulation and the regulon of the general stress regulator σ^B in *B. cereus* differ from that in *B. subtilis*. Besides the much smaller regulon of σ^B in *B. cereus*, the σ^B regulons of *B. subtilis* and *B. cereus* share only eight genes (van Schaik *et al.*, 2007). *B. cereus* has more transcriptional regulators belonging to the sigma factor-family possibly for fine tuning the stress response (van der Voort, 2008). These differences together with the fact that *B. cereus* and *B. subtilis* are not that closely related (Xu and Cote, 2003) signifies the importance of studying *B. cereus* and related organisms separately and that it will not be possible to extrapolate all data gained on the Gram-positive model organism *B. subtilis* to *B. cereus* and related species.
The utilization of nutrients, the predictions based on genome sequences and the relation of *B. cereus* to other *Bacillus* species contributed to the discussion about the niche of *B. cereus*. Soil, where *B. cereus* occupies a saprophytic lifestyle, and insects, where *B. cereus* is present in the gut as a commensal organism, are thought to be the main niches of *B. cereus* (Jensen *et al.*, 2003; Stenfors Arnesen *et al.*, 2008). *B. cereus* is capable of acquiring additional genetic information by taking up plasmids and/or horizontal gene transfer, indicated by the large diversity in plasmids found in *B. cereus* sensu lato strains. The genetic information contained in these plasmids and other transferable genetic elements may further contribute to the successful occupation of a variety of environments by *B. cereus* group members.

Acid stress response

B. cereus can persist in environments that have a low pH, such as soil and plant rhizosphere, which can be as low as pH 4 due to exudation of protons and organic acids by plants (Neumann and Martinoia, 2002). Also various kinds of foods may have a low pH either due to fermentation or the addition of organic acids that are used to control bacterial growth. However, most foods, which have been associated with outbreaks of *B. cereus*, generally do not have a low pH. For instance, rice and pasta have a pH of approximately pH 6.0, the pH of milk is pH 6.3 or higher, and most vegetables have a pH around 6.0 but can be as low as pH 4.0 (Bad Bug Book, Food and Drug Administration, USA, website: http://www.fda.gov/Food/FoodSafety/FoodborneIllness/FoodborneIllnessFoodbornePathogensNaturalToxins/BadBugBook/ucm122561.htm). Furthermore, upon ingestion of food contaminated with *B. cereus*, the organism has to survive gastric passage in order to reach the intestines. Generally, the pH of the stomach may be as low as pH 2, however elderly people have a higher stomach pH and also drug usage and food intake may elevate the pH. Studies simulating and modelling gastric passage suggest that a large population of the ingested vegetative cells from *B. cereus* may survive the gastric passage, dependent on the

growth phase of the vegetative cells, the type of strains, and the age of the consumer (Clavel *et al.*, 2004; Wijnands *et al.*, 2009). Taken together, all these aspects point to an important role of acid resistance in the pathogenicity of *B. cereus*, and an apparent highly relevant subject to study.

Up to this thesis (Chapter 3, Chapter 4, Chapter 5), only limited information was available about the low pH response of *B. cereus*. It was previously shown that *B. cereus* displays acid adaptation at pH 6.3, resulting in enhanced protection when subsequently exposed to pH 4.6, heat, ethanol, salt, or hydrogen peroxide. This acid tolerance response was associated with maintenance of the internal pH and a role for superoxide dismutase was suggested (Browne and Dowds, 2002; Jobin *et al.*, 2002). Furthermore, acid tolerance was shown to be dependant on de novo protein synthesis (Thomassin *et al.*, 2006). Mechanisms of acid resistance have been studied more extensively in other Gram-positive organisms and were reviewed by Cotter and Hill (2003). Most studies were performed on the acid stress response of non-respiring lactic acid bacteria in the presence of oxygen. In contrast, *B. cereus* actively respires in the presence of oxygen and therefore the results obtained from these lactic acid bacteria should only be extrapolated cautiously to *B. cereus*.

Besides differences between the organisms reviewed by Cotter and Hill (2003) and *B. cereus*, there are mechanisms described to be involved in acid stress that are also present in *B. cereus* and putatively play a role in low pH exposures as indicated by induction of the corresponding genes (Fig. 1). Genes involved not only in acid stress response but also in responses to other stresses, including protein repair chaperones *groESL* and *dnaK* and *clp* genes, were shown to be up-regulated upon exposure to acid conditions in *B. cereus*. Furthermore, several transcriptional regulators are putatively involved in the acid stress response. The expression of *sigB*, the gene encoding for alternative sigma factor σ^B, was induced, which is in agreement with previous studies (van Schaik *et al.*, 2004b). Heat stress regulators *ctsR* and *hrcA* (van de Guchte *et al.*, 2002) were also up-regulated upon exposure to low pH in *B. cereus*, indicating possible common damaging factors in different stress conditions.

Metabolic rearrangements, known to be involved in acid resistance in lactic acid bacteria, were also induced by *B. cereus* upon low pH exposures. Genes encoding for enzymes catalyzing the reaction from pyruvate to acetoin and butanediol, i.e., *alsDS*, were induced upon exposure to acid shocks. Although such reaction is at the expense of pyruvate, it removes intracellular proteins and forms carbon dioxide (CO_2). Also in *B. subtilis alsSD* genes are strongly induced under mild acid stress conditions (Wilks *et al.*, 2009) and in *Lactobacillus plantarum* activation of the corresponding enzymes contributed to pH homeostasis (Tsau *et al.*, 1992). Genes encoding for alcohol dehydrogenases and lactate dehydrogenases were induced upon exposure to lethal acid shocks. Therefore, the conversion of pyruvate to ethanol or lactate, generating CO_2 and dissipating H^+, may be an ultimate futile response of *B. cereus* to deal with low intracellular pH (pH_i) or restoration of NAD^+/NADH balance. Some metabolic rearrangements were found specifically correlated with lactic acid or acetic acid stress, but their functions remain to be established.

Metabolomics and mutant analysis will aid in unravelling their role in organic acid resistance.

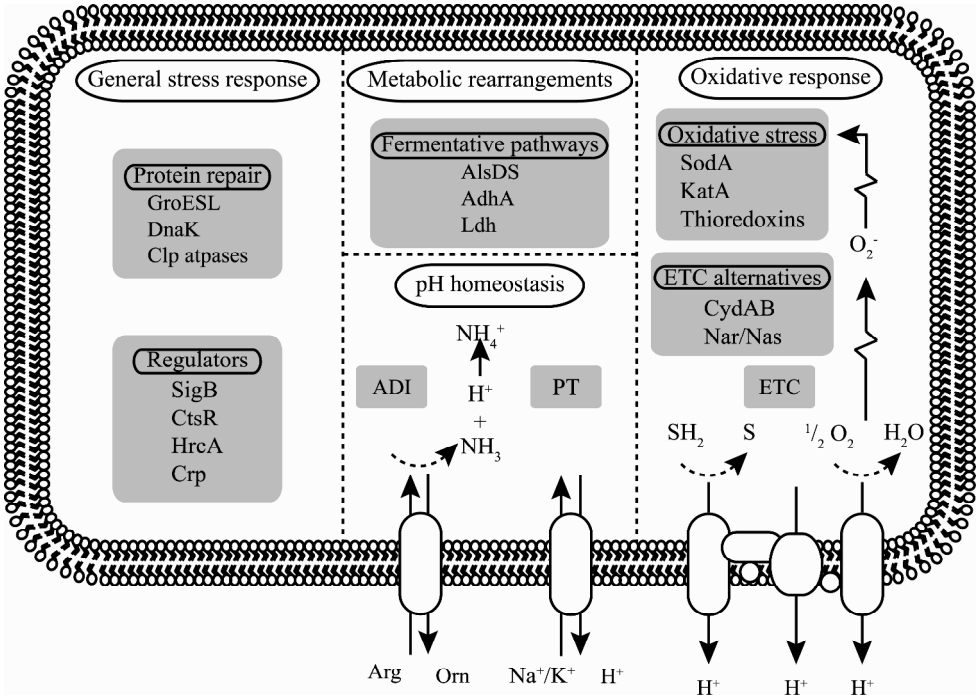

Fig. 1. Graphical representation of general acid stress associated mechanisms in *B. cereus* divided in four different groups: (i) general stress response, (ii) metabolic rearrangements, (iii) pH homeostasis, and (iv) oxidative response. The general stress response group involves genes that are putatively not only induced by low pH, but may be involved in a more general response to stresses. The transcription of protein repair mechanisms, including the chaperones GroESL and DnaK and the Clp proteases, as well as several transcriptional regulators, such as σ^B (SigB), CtsR, HrcA, and Crp, was changed upon low pH exposure. The most notable metabolic rearrangements shown upon exposure to mainly organic acid stress were fermentative pathways, such as acetoin production (AlsDS), alcohol (AdhA) and lactate dehydrogenases (Ldh) and rerouting of pyruvate metabolism. pH homeostasis involves proton dependant transporters (PT) that may transport protons inwards and outwards. A mechanisms of proton consumption induced upon low pH exposure in *B. cereus* was the arginine deiminase (ADI) pathway. Oxidative response may not be directly involved in the resistance to acid stress, however, genes involved in oxidative stress were shown to be heavily up-regulated upon exposure to low pH. The electron transfer chain (ETC) is conceivably disturbed by a low pH, generating superoxide. Superoxide can lead to the formation of other reactive oxygen species and may induce oxidative stress mechanisms, including thioredoxins, catalase (KatA), and superoxide dismutase (SodA). Furthermore, the perturbation of the ETC is corroborated by the expression of alternatives for the ETC, such as cytochrome d ubiquinol oxidase (CydAB) and nitrate/nitrite reductase (Nar/Nas). Other abbreviations used: S: substrate, Arg: arginine, Orn: ornithine

Upon exposure to acid conditions, many bacteria activate enzymes contributing to pH homeostasis. Cells may pump protons out of the cell, prevent protons from leaking in, and counteract acidification of the cytoplasm by producing alkaline compounds. Aerobic bacteria, such as *B. cereus*, use their electron transport machinery to transport protons over the cell membrane generating an excess of protons on the outside of the cell thus generating a proton motive force (PMF). The PMF is subsequently used to generate ATP by inward flux of protons via F_1F_0-ATPase. Lactic acid bacteria F_1F_0-ATPase can also transport protons outside the cell at the expensive of ATP in acid conditions. *B. cereus* represses the expression of genes encoding for subunits of F_1F_0-ATPase upon exposure to mild acidic environments. Conceivably, *B. cereus* does not use F_1F_0-ATPase to pump protons out of the cell in acid conditions and by repressing F_1F_0-ATPase genes and lowering the amount of active ATPase, the influx of protons is diminished. Notably, upon exposure to lethal levels of acidity these genes are not repressed. Also other proton transporters, such as *napA* and *nhaC*, were down-regulated upon exposure to mild acid stress. Interestingly, these genes were (highly) induced upon exposure to lethal pHs, indicating a fine balance between ATP synthesis and the regulation of pH_i. This is corroborated with the loss of ATP upon exposure to lethal levels of acetic acid (Chapter 4). Amino acid decarboxylases are used by bacteria to regulate their pH_i. In Gram-positives, especially *Listeria monocytogenes*, glutamate decarboxylase (GAD) has been associated with acid resistance (Cotter *et al.*, 2001). Glutamate is converted to gamma-aminobutyric acid (GABA) consuming an intracellular proton by GAD. Subsequently, the product GABA is exchanged with extracellular glutamate by a glutamate/GABA antiporter. Such transporter has been found to be necessary for optimal GAD-dependant acid resistance in *L. monocytogenes*. In *B. cereus* ATCC 14579 no GAD system could be identified on its genome. In contrast, ATCC 10987 does harbour a glutamate decarboxylase gene (Chapter 2) that was however not found to be differentially expressed upon exposure to low pHs (Chapter 5), indicating that the role of GAD in the acid resistance of *B. cereus* ATCC 10987 is limited. An explanation for this phenomenon is the fact that a glutamate/GABA antiporter gene is lacking in the genome of ATCC 10987. Bacteria can also counteract a low internal pH by the production of alkaline compounds, such as ammonia. One of the mechanisms known to produce ammonia and involved in acid resistance in other bacteria is the arginine deiminase pathway (ADI) (Ryan *et al.*, 2009). The ADI pathway converts arginine into ammonia and CO_2 via citrulline and carbamoyl-phosphate. Although the role of ADI in acid resistance in other bacteria is evident, in *B. cereus* the ADI genes are only moderately up-regulated upon exposure to acid in aerobic conditions, whereas the ADI pathway was found highly up-regulated under mildly acidic anaerobic conditions, suggesting that this system may play a role in acid stress survival in anaerobic conditions (van der Voort and Abee, 2009). Arginase, that also converts arginine to citrulline producing ammonia, was highly induced upon exposure to low pHs, indicating that arginine catabolism may support acid tolerance in *B. cereus*. Another well-known mechanism of alkali production is the hydrolysis of urea into ammonia and CO_2 by the enzyme urease. Urease is known to be involved in the acid

resistance of several bacteria and well-studied in *Helicobacter pylori* and *B. subtilis* (Mobley *et al.*, 1995; Wray *et al.*, 1997). Urease and concomitant ureolytic activity is shown by ATCC 10987, in contrast to ATCC 14579, which does not harbour the urease genes. The genes encoding for the urease enzyme were somewhat induced upon exposure to sub-lethal pH 5.4 in ATCC 10987 (Chapter 5). However, it was shown that the ureolytic activity in a variety of *B. cereus* strains, including ATCC 10987, did not provide for acid resistance and that its role was solely in nitrogen metabolism (Chapter 3). Furthermore, the expression of urease was found to be very low and not up-regulated by acid. The different results observed in chapters 3 and 5 may originate from the different techniques used. The urease encoding mRNA is present close to one copy per cell. When up-regulated just a few times, Q-PCR analysis will not give significant results because the number of mRNA molecules of urease genes is normalized on the basis of highly copied housekeeping genes, such as *tufA* and *rpoA*. With the microarray analysis, on the other hand, the urease mRNA of a reference condition is compared to that of a sample condition not regarding the expression of single highly expressed housekeeping genes. Therefore, the expression of urease may be induced upon exposure to low pHs, however, the concomitant ureolytic activity is not sufficient to provide for acid resistance in *B. cereus* ATCC 10987.

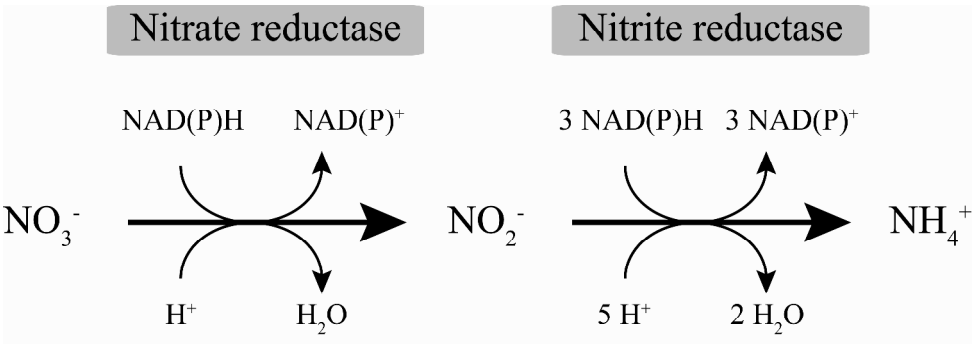

Fig. 2. Schematic representation of nitrate and nitrite reduction to ammonium performed by nitrate and nitrite reductase, respectively.

The exposure of *B. cereus* to inorganic acid as well as organic acids, lactic acid and acetic acid, revealed a major oxidative response (Chapter 4 and Chapter 5). The oxidative response was indicated by the induction of oxidative stress associated genes, including genes encoding for thioredoxins, catalases, superoxide dismutase and the major oxidative stress regulator PerR, upon exposure to acid shocks. Thioredoxins are known to control the reduced state of thiol groups that can be oxidized upon exposure to oxidative stress (Holmgren, 1985). Superoxide dismutase and catalase convert superoxide and hydrogen peroxide into water (Imlay, 2003). PerR is a hydrogen peroxide sensing transcriptional regulator associated with the regulation of catalases and peroxidases (Mongkolsuk and

Helmann, 2002). The induction of these oxidative stress-associated genes suggests that oxidative compounds are generated upon exposure to low pHs in *B. cereus*. Reactive oxygen species may be generated at specific sites in the aerobic electron transfer chain (ETC). The ETC activity may have been affected by acid shock as expression of genes encoding alternative electron donor and acceptor mechanisms was induced. Most prominent was the induction of the ATCC 14579-specific nitrate and nitrite reductase cluster upon exposure to acid shocks (Chapter 2 and Chapter 5). Nitrate can act as an alternative electron acceptor and is converted to nitrite in a reaction consuming a proton (Richardson *et al.*, 2001). Subsequently the resultant nitrite can be reduced to ammonium using five protons (Fig. 2). Whether nitrate and nitrite reductases are induced because they form an alternative ETC, restore NAD^+/NADH balance, and/or because the reactions they catalyze consume intracellular protons remains to be elucidated. Also other alternative components of the ETC were associated with mainly lethal levels of organic and inorganic acid shocks (Chapter 4 and Chapter 5). Cytochrome *bd* oxidase (*cydAB*) genes, which may act as an alternative complex IV of the ETC was highly induced upon exposure to 15 mM acetic acid and at pH 4.5. Cytochrome *bd* oxidase has been proposed to function as an alternative electron transport chain in concert with NAD(P)H dependant dehydrogenases, such as lactate (*ldh*) and alcohol dehydrogenase (*adhA*) (Chai *et al.*, 2009). Lactate dehydrogenase (*ldh*) and cytochrome *bd* oxidase genes are co-ordinately expressed together with the lactate permease gene *lctP* and formate-nitrite transporter gene *ywcJ* and under control of the negative regulator YdiH (Rex) in *B. subtilis* (Larsson *et al.*, 2005). Together with the *alsSD* genes, *cydAB*, *ldh*, and *lctP* form a distinct regulon, which is part of the larger Fnr regulon (Reents *et al.*, 2006), indicating a clear association between these up-regulated genes and anaerobic conditions. Whether activation of these enzymes and pathways contribute to higher acid resistance of *B. cereus* when grown and exposed without oxygen remains to be elucidated (Chapter 6).

The different responses observed in our studies are in line with observations on the acid tolerance response of *B. cereus* pre-exposed to mild low pHs, providing protection to lethal pH conditions (Jobin *et al.*, 2002; Thomassin *et al.*, 2006). Furthermore, this thesis provides detailed information about the mechanisms involved in low pH resistance, including factors that may contribute to cross protection to other stresses as discussed below. Acid stress exposure and concomitant oxidative response may have an impact on the pathogenic potential of *B. cereus*. Genes encoding phospholipase C and cytotoxin K were induced upon exposure to low pH. Thus, the low pH environment of the human stomach and the small intestine pH 6 may induce virulence factors in *B. cereus*. Furthermore, the oxygen limited environment of the small intestine also induces toxin formation and increases the resistance to low pH of *B. cereus* (Zigha *et al.*, 2006). To conclude, surviving gastric passage may have prepared *B. cereus* cells to cope more efficient with the adverse conditions encountered in the intestinal tract thus increasing its virulence potential.

Radical formation

Radicals play an important role in all sorts of biological processes. Radicals act as signal transducers in cell-signalling in animals and plants. Plants generate reactive oxygen species (ROS) to control various processes, including pathogen defence, programmed cell death, and stomatal behaviour (Apel and Hirt, 2004). In animals, ROS may influence cell proliferation, cell death (either apoptosis or necrosis) and the expression of genes, and may be involved in the activation of several signalling pathways, activating cell signalling cascades (Genestra, 2007). Furthermore, ROS have been associated with aging and diseases in humans, including Parkinson, Alzheimer, heart failure, diabetes, and cancer (Kang and Hamasaki, 2005). In bacteria, radicals may also play an important role in various conditions shown by the induction of mechanisms that protect against oxidative damage. Genes involved in oxidative stress are commonly induced in various bacterial species in response to exposure to different environmental conditions. For example, activation of oxidative stress genes has been associated with exposure to, heat, cold plasma, cell envelope perturbing chemicals, and acid in Gram-negative and Gram-positive organisms (Fleury *et al.*, 2009; Sharma *et al.*, 2009; Sikora *et al.*, 2009). Furthermore, the response of *Escherichia coli* and *Salmonella* under conditions mimicking natural habitats as the human gastro-intestinal tract and food shows parallels with the response displayed upon exposure to oxidative stress (Rychlik and Barrow, 2005; Bergholz *et al.*, 2009). Also in *B. cereus* the relation between oxidative stress genes and other adverse conditions has been noted. Upon exposure to heat, σ^B-dependant catalase expression increased in *B. cereus* (van Schaik *et al.*, 2004a; van Schaik *et al.*, 2007). Catalase activity and the expression of oxidative stress related genes, such as superoxide dismutase is increased in *B. cereus* upon exposure to salt and bile salt stress (Kristoffersen *et al.*, 2007; den Besten *et al.*, 2009).

The induction of oxidative stress associated genes and ETC alternatives upon exposure to low pH shocks and the recent discovery that bactericidal antibiotics induced radical formation in *E. coli* and *Staphylococcus aureus* (Kohanski *et al.*, 2007) lead to the discovery of acid induced inactivation-associated radical formation (Chapter 5). In Chapter 6, the formation of hydroxyl radicals (OH·) and/or peroxynitrite ($ONOO^-$) was also shown for lethal heat exposure of *B. cereus* strains ATCC 14579 and ATCC 10987 using the fluorescent probe 3'-(p-hydroxyphenyl) fluorescein (HPF) that targets both radicals. OH· can be formed by the reaction of hydrogen peroxide and free ferrous iron, the Fenton reaction (Fig. 3). In this reaction ferrous iron (Fe^{2+}) is oxidized to ferric iron (Fe^{3+}). Reduced thiol groups can reduce Fe^{3+} back to Fe^{2+} resulting in oxidized thiol groups. Thioredoxins, of which several are induced by *B. cereus* upon acid stress exposure (Chapter 5, *Supplementary material*), reduce oxidized thiol groups. Nitric oxide (NO), putatively formed by nitric oxide synthase, inhibits thioredoxins (Gusarov and Nudler, 2005). NO also activates catalase activity that converts hydrogen peroxide into water and oxygen. Thus NO inhibits OH· formation by removing H_2O_2 and Fe^{2+} from the Fenton reaction (Sudhamsu and Crane, 2009) and blocks O_2^- formation by inhibiting the aerobic ETC (Husain *et al.*, 2008). A drawback to the NO-derived protective effect against OH· formation is that NO

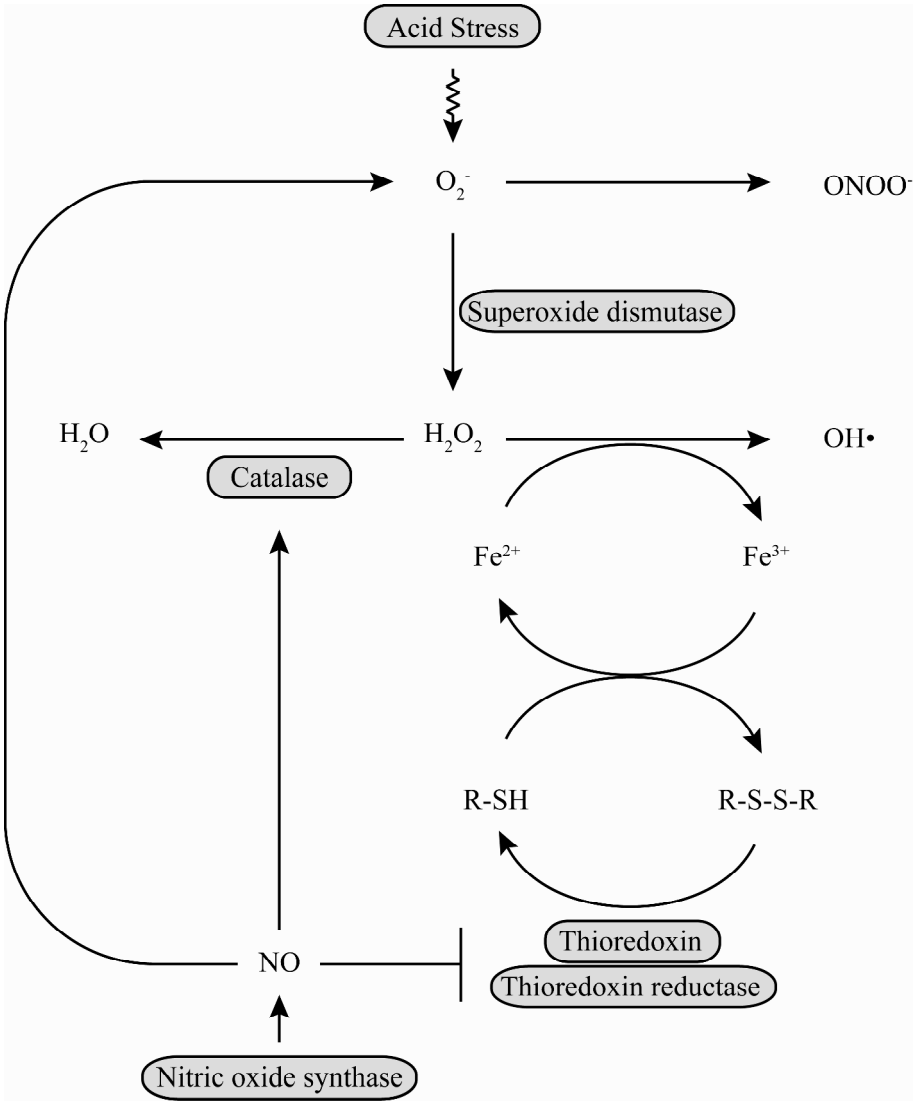

Fig. 3. Schematic representation of the putative role of nitric oxide (NO) in the formation of hydroxyl radicals (OH·) and peroxynitrite ($ONOO^-$). NO inhibits the formation of reduced thiols (R-SH) that recycle Fe^{3+} to Fe^{2+} and, thus, inhibits the OH· forming Fenton reaction. NO also activates catalase that breaks down hydrogen peroxide (H_2O_2) to oxygen and water (H_2O). A drawback to these protective mechanisms of NO is that NO itself may react with superoxide (O_2^-) to form highly reactive $ONOO^-$.

can react with superoxide to form another highly reactive ROS: $ONOO^-$. A crucial role in the development of $OH\cdot$ and $ONOO^-$, is the formation of O_2^-. Besides reacting with NO to $ONOO^-$, O_2^- can be converted to H_2O_2 by superoxide dismutase and O_2^--initiated damage of iron-sulphur clusters can make Fe^{2+} available for the Fenton reaction (Kohanski *et al.*, 2007). The generation of O_2^- was firstly indicated by the induction of superoxide dismutase genes in *B. cereus* ATCC 14579 and ATCC 10987 upon exposure to acid shocks (Chapter 4 and Chapter 5). In Chapter 7, O_2^- formation was shown for *B. cereus* ATCC 14579 upon exposure to heat and acid with the fluorescent probe MitoSOX. O_2^- is conceivably generated at the ETC, where free electrons prematurely leak to oxygen (Robinson *et al.*, 2006). The formation of O_2^- was not dependant on membrane damage (data not shown), but was dependant on the presence of oxygen, which is in concert with the results obtained in Chapter 6 showing that $OH\cdot$ and/or $ONOO^-$ formation was also dependant on oxygen availability.
In chapter 7, O_2^- formation has not only been shown for *B. cereus* strains ATCC 14579 and ATCC 10987, but also for *B. subtilis*, *L. monocytogenes*, *E. coli*, and *Salmonella* Typhimurium upon exposure to heat. Together with the commonly induced oxidative stress associated genes, this suggests that oxidative stress and formation of ROS commonly play role in bacterial response to various adverse conditions. However, the contribution of reactive oxygen and nitrogen species to the actual inactivation of the bacterial cells remains to be resolved and would be an interesting subject to investigate. Newly developed methods, such as microsensors that are used in marine microbiology for analysis of the nitrogen cycle (Schreiber *et al.*, 2008) and the use of specific chemicals that act as inhibitors of ETC components, may be used to unravel the details of acid-induced radical formation (Robinson *et al.*, 2006).

Concluding remarks

The aims of this thesis and of the overall Food Safety and Preservation project of TIFN were to identify key factors and Achilles' heels in stress responses and indicators of physiological states, and to provide mechanistic understanding of stress responses. The approach used in this study, combining genotypic and transcriptomic data with physiological responses and measurements of intracellular ROS, provided detailed information on the acid stress responses of *B. cereus*. Key factors, including nitrate and nitrite reductases, Achilles' heels, such as the formation of ROS, and biomarkers, for example *dnaK*, were revealed. Furthermore, the concurrent analysis of two strains and multiple stress levels enables for distinguishing between phenotype-specific, stress level-specific and strain-specific transcriptome responses. This approach showed not only the well-studied responses to mild pHs, including the induction of several general stress response genes, but also the response to lethal levels of acidity, an issue that has up to now mostly been neglected. Although microarrays only allow for investigating an entire bacterial population, combining such analysis with flow cytometry and fluorescence microscopy allowed for the discovery of inactivation-associated radical formation in single

cells. The formation of highly ROS, such as hydroxyl radicals, peroxynitrite, and superoxide, upon heat and acid exposures was shown to be dependant on the presence of oxygen. This corresponded with the higher resistance of *B. cereus* when grown and exposed in absence of oxygen, suggesting that highly reactive oxygen and nitrogen species may play a role in inactivating *B. cereus* upon stress exposure. To study this role and to investigate if the secondary oxidative response is a common mechanism of cellular death in bacteria exposed to severe aerobic stress conditions a new method, using the fluorescent probe MitoSOX that allows for superoxide detection in bacteria, was designed. The use of MitoSOX will allow for further studies on the role of ROS in bacterial stress responses and the identification of corresponding adaptation and survival strategies. The combination with flow cytometry warrants an efficient high throughput analysis enabling assessment of population heterogeneity in O_2^- formation and assessment of its impact on other relevant biological parameters. Therefore, the findings in this study can contribute to further understanding of bacterial stress responses and secondary oxidative responses. Furthermore, the results obtained may aid to optimize and select (combinations of) stresses to apply in hurdle technology, thus enabling design of safe, milder food processing and preservation techniques.

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Samenvatting

Bacillus cereus is een bacterie die van nature bijna overal voorkomt zo ook in verschillende typen voedsel, zoals melk, pasta en rijst. In voedsel kan hij voor bederf zorgen, maar ook ziekteverwekkend. *B. cereus* kan twee verschillende typen van voedselvergiftiging veroorzaken, te onderscheiden door de verschillende symptomen, overgeven en diarree. Overgeven wordt veroorzaakt door een toxine dat sommige stammen van *B. cereus* in voedsel kunnen produceren. Dit toxine, genaamd cereulide, wordt niet afgebroken door koken en wordt na vorming geconsumeerd met het voedsel. *B. cereus* geassocieerde diarree wordt veroorzaakt door groeiende cellen in de dunne darm van de mens. De bacteriecellen maken tijdens de groei zogenoemde enterotoxines. Voordat *B. cereus* in de darm kan groeien, moet hij het zure milieu (pH 2 - 4) van de maag overleven. Er zijn ruwweg twee manieren die *B. cereus* in staat stellen de maag te passeren. *B. cereus* kan ongevoelige overlevingscapsules, ook wel sporen genoemd, vormen of *B. cereus* kan zich aanpassen in mild zure condities (pH 5 - 6) wat ervoor zorgt dat de cellen het verblijf in de maag kunnen overleven. Mild zure condities zijn bijvoorbeeld te vinden in voedsel, zoals sauzen en groenten, van waaruit na nuttiging robuustere *B. cereus* cellen de maag in komen. Hoe *B. cereus* zich aan kan passen aan zure condities is beschreven in dit proefschrift.

We hebben in het onderzoek beschreven in dit proefschrift twee verschillende stammen onderzocht, omdat verschillende *B. cereus* stammen zeer uiteenlopende eigenschappen kunnen hebben. De precieze verschillen op het gebied van erfelijke informatie en groeicapaciteit tussen deze twee stammen, *B. cereus* ATCC 14579 en ATCC 10987, hebben we onderzocht. Uit dit onderzoek, wat beschreven is in hoofdstuk 2, is gebleken dat, ondanks de nauwe verwantschap van alle *B. cereus* stammen, de twee onderzochte stammen zeer verschillend waren, dat wil zeggen ongeveer 20% van de genen van deze stammen zijn verschillend. Er zijn mogelijke aanwijzingen gevonden tussen deze stamspecifieke genen en de capaciteit van de stammen om op bepaalde substraten te groeien. De aanpak om genoom breed te zoeken naar stamspecifieke genen en deze te koppelen aan verschillende groei-eigenschappen van stammen was nieuw en kan een basis vormen voor verder onderzoek.

Eén van de verschillen die in hoofdstuk 2 naar boven kwam was de aanwezigheid van een ureasecluster op het genoom van *B. cereus* ATCC 10987. In hoofdstuk 3 is de aanwezigheid en de rol van dit cluster in de zuuroverleving onderzocht in 49 *B. cereus* stammen. Urease is een enzym dat ureum, een veel voorkomend afvalproduct in het menselijk lichaam, omzet in ammoniak en koolstofdioxide. In potentie kan ammoniak gebruikt worden door bacteriën om zure condities te overleven doordat zuur kan worden weggevangen waarbij ammonium gevormd wordt. In 10 van de onderzochte 49 *B. cereus* stammen is de aanwezigheid van ureasegenen aangetoond en 8 van deze stammen waren in staat om ureum te gebruiken als substraat om te groeien. Geen van de ureasepositieve stammen waren met of zonder ureum beter in staat om zure condities te overleven dan

stammen die geen ureaseenzym hadden. De expressie en de activiteit van het urease bleek te laag te zijn om voor verhoogde zuurresistentie te zorgen. Dit onderzoek toonde dus aan dat urease niet werd gebruikt door *B. cereus* om zure condities te overleven.
Wat *B. cereus* wel gebruikt om zure condities het hoofd te bieden is beschreven in hoofdstuk 4. In dit hoofdstuk is onderzocht hoe *B. cereus* reageert op een verlaagde pH, pH 5.5, bewerkstelligd door middel van het toevoegen van anorganisch zuur, zoals zoutzuur (HCl), organisch zuren, zoals azijnzuur (HAc) en melkzuur (HLa), of een combinatie van HCl en HAc. De groei van *B. cereus* werd door blootstelling aan HCl geremd en door HAc/HCl en HLa gestopt. Blootstelling aan HAc leidde, naast een groeistop, na langere incubatie tot afsterving van de cellen. Dit verschil in fysiologische response tussen de verschillende blootstellingen wordt mogelijk door twee factoren veroorzaakt, door de extra inhiberende werking van de organische zuren en door de verschillende concentraties van het non-gedissocieerde organische zuur. Door middel van metingen van ATP, de algemene energiedrager in de bacteriecel, is gebleken dat de verschillende fysiologische responsen die te zien zijn na de verschillende behandelingen goed correleren aan de energieniveaus in de cellen. Daarbij hadden de groeiende cellen (HCl-blootstelling) een hoog energieniveau en de stervende cellen (HAc-blootstelling) een laag energieniveau. De genexpressieanalyse, die laat zien welke genen er aan of uit gaan tijdens een bepaalde behandeling in de bacteriecellen, liet zien dat de expressie van genen betrokken bij verschillende metabole routes, zoals omzettingen van pyruvaat en butaandiol, veranderde onder alle geteste zuurcondities en dat een aantal fermentatieve routes, waarin lactaat, formaat of ethanol gevormd worden, alleen geïnduceerd werden tijdens blootstelling aan de dodelijke HAc concentraties.
Het verschil in fysiologische response tussen de verschillende mate van zuurblootstelling en de bijbehorende genexpressies zijn verder onderzocht in hoofdstuk 5, waarbij gekeken is naar het effect van verschillende zuurgraden (pH 5.4 - pH 4.4), bewerkstelligd door middel van het toevoegen van HCl, op de twee model *B. cereus* stammen ATCC 14579 en ATCC 10987. *B. cereus* ATCC 14579 was beter bestand tegen lage pHs dan ATCC 10987, en kon pH's tussen 5.0 en 4.7 overleven (zonder af te sterven of te groeien) terwijl ATCC 10987 al wel werd geïnactiveerd bij deze pH's. Genexpressieanalyse van beide stammen bij pH 5.4, pH 5.0, pH 4.8, of pH 4.5 liet overeenkomstige en stamspecifieke aanpassingen zien aan het zure milieu. De grote inductie in beide stammen van genen die geassocieerd zijn met blootstelling aan oxidatieve stoffen in alle condities viel op en leidde tot het onderzoek naar de vorming van zeer reactieve moleculen, genaamd radicalen. De vorming van radicalen (hydroxyl en peroxynitriet) kon alleen aangetoond worden in beide stammen onder condities die lethaal waren voor de cellen. Aan de hand van deze resultaten is een model geformuleerd dat kan verklaren hoe deze radicalen worden gevormd wanneer de cellen blootgesteld worden aan letale zure condities.
In dit model speelt de vorming van superoxide uit zuurstof een cruciale rol. In hoofdstuk 6 is daarom onderzocht of de aanwezigheid van zuurstof invloed heeft op de resistentie van *B. cereus* ATCC 14579 en ATCC 10987 blootgesteld aan een hoge temperatuur, zuur, hoge

zoutconcentraties en waterstofperoxide. Cellen van beide stammen waren beter bestand tegen een hoge temperatuur en zuur wanneer deze cellen gekweekt en blootgesteld werden in afwezigheid van zuurstof. De zoutresistentie veranderde niet significant door de aan- of afwezigheid van zuurstof, maar cellen gegroeid en blootgesteld met zuurstof waren beter bestand tegen waterstofperoxide. Deze resultaten kwamen overeen met de vorming van hydroxyl en/of peroxynitriet tijdens de blootstelling aan hoge temperaturen en zuur in aanwezigheid van zuurstof. Zout en waterstofperoxide induceerden geen overmatige vorming van hydroxyl en/of peroxynitriet. Ook de blootstelling aan een hoge temperatuur in afwezigheid van zuurstof leverde geen aantoonbare vorming op van de genoemde radicalen. Hieruit kon dus geconcludeerd worden dat de verhoogde inactivatie van aeroob gegroeide *B. cereus* cellen na blootstelling aan zuur en hitte overeenstemde met de vorming van zeer reactieve radicalen zoals hydroxyl en peroxynitriet en dat deze resultaten aantonen dat de aan- of afwezigheid van zuurstof de inactivatieefficiëntie van conserveringsstappen kan beïnvloeden.

Om de vorming van superoxide tijdens blootstelling aan hitte en zuur door *B. cereus* te onderzoeken is een methode ontwikkeld die gebruikt maakt van de fluorescente probe MitoSOX. MitoSOX is een probe die na een reactie met superoxide en de binding aan DNA een rood fluorescent signaal geeft. Wanneer dit rode signaal te zien is (of gemeten kan worden door middel van een flowcytometer) in de cellen onder een fluorescentie microscoop, dan hebben de cellen superoxide gevormd. *B. cereus* maakt superoxide na aerobe blootstelling aan hitte, zuur en telluriet. Deze methode werkt ook in andere bacteriën zoals de Grampositieve modelbacteriën *Bacillus subtilis* en *Listeria monocytogenes* en de Gramnegatieve modelbacteriën *Salmonella* Typhimurium en *Escherichia coli*. Deze methode kan dus gebruikt worden voor verder stressonderzoek in bacteriën.

Het doel van het onderzoek beschreven in dit proefschrift en dat van het "Food Safety and Preservation" project van het Top Institute Food and Nutrition in zijn algemeenheid was om belangrijke factoren van de bacteriële stressresponse en zijn eventuele zwakke punt, de "Achilles hiel", te identificeren en om een mechanistisch begrip te krijgen van de stressresponse. De aanpak van het onderzoek beschreven in dit proefschrift, het combineren van informatie uit de genetische blauwdruk van *B. cereus* bacteriën, genexpressieprofielen en fysiologische analyses hebben gedetailleerde informatie opgeleverd over de response van zuurblootgestelde *B. cereus*. Belangrijke factoren, zoals de nitraat- en nitrietreductases van *B. cereus* ATCC 14579, de vorming van zeer reactieve radicalen en biomarkers, zoals dnaK konden worden geïdentificeerd. Deze aanpak liet niet alleen de response van *B. cereus* zien ten aanzien van milde zuur stress, maar ook de response naar aanleiding van letale zuurniveaus, iets wat tot nu toe onderbelicht is gebleven, waardoor de impact van radicaalvorming tijdens stressblootstelling aan het licht is gekomen. De resultaten beschreven in dit proefschrift kunnen bijdragen aan het verder ontrafelen van de bacteriële stressresponse inclusief de secundaire oxidatieve response die *B. cereus* en mogelijk ook andere bacteriën laten zien wanneer de cellen worden blootgesteld aan letale stressniveaus. Verder kunnen de resultaten gebruikt worden om verschillende stressen (of combinaties

daarvan) te selecteren en te optimaliseren, waardoor het mogelijk zal zijn nog mildere, maar toch ook veilige, voedingsverwerking- en conserveringstechnieken te ontwerpen.

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Finally, after five years, this thesis has come to an end. Obviously I could not have accomplished this mission without the help of others. Therefore, I would like to take this opportunity to acknowledge several people.
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Along the way of conducting the research for my PhD thesis, I supervised (together with Tjakko) a few BSc and MSc students. Arend and Joop both worked on a topic which is, unfortunately, not included in this thesis. Anyway, thanks for your interest and help. Ilona, your project was more successful and resulted in chapter 6. Your help was indispensable, thanks.
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List of Publications

R. Voulhoux, M.P. Bos, J. Geurtsen, **M. Mols**, J. Tommassen, 2003, Role of a highly conserved bacterial protein in outer membrane protein assembly, Science, Vol. 299, No. 5604, 262-265.

A.P.H.M. Hermans, **M. Mols**, P.A. Berk, H.J.M. Aarts, M.H. Zwietering, T. Abee, 2007, Comparative transcriptome analysis of stress and virulence genes in *Salmonella enterica* serovar Typhimurium DT104 wild type and its *luxS* deletion mutant at various growth phases, In: Stress response and virulence in *Salmonella* Typhimurium: a genomics approach, PhD Thesis A.P.H.M. Hermans, Wageningen University.

M. Mols, M. de Been, M.H. Zwietering, R. Moezelaar, T. Abee, 2007, Metabolic capacity of *Bacillus cereus* strains ATCC 14579 and ATCC 10987 interlinked with comparative genomics, Environmental Microbiology, Vol. 9, No. 12, 2933-2944.

M. Mols, T. Abee, 2008, Role of ureolytic activity in *Bacillus cereus* nitrogen metabolism and acid survival, Applied and Environmental Microbiology, Vol. 74, No. 8, 2370-2378.

H.M. den Besten, **M. Mols**, R. Moezelaar, M.H. Zwietering, T. Abee, 2009, Phenotypic and transcriptomic analyses of mildly and severely salt-stressed *Bacillus cereus* ATCC 14579 cells, Applied and Environmental Microbiology, Vol. 75, No. 12, 4111-4119.

M. Mols, R. van Kranenburg, M.H. Tempelaars, W. van Schaik, R. Moezelaar, T. Abee, Comparative analysis of transcriptional and physiological responses of *Bacillus cereus* to organic and inorganic acid shocks, *submitted for publication*.

M. Mols, R. van Kranenburg, C.C.J. van Melis, R. Moezelaar, T. Abee, Comparative transcriptome and phenotype analysis of acid-stressed *Bacillus cereus* strains ATCC 14579 and ATCC 10987 reveals a major oxidative response and inactivation associated radical formation, *submitted for publication*.

M. Mols, I. Pier, M.H. Zwietering, T. Abee, The impact of oxygen availability on stress survival and radical formation of *Bacillus cereus*, *accepted for publication in International Journal of Food Microbiology*.

M. Mols, M. Ceragioli, T. Abee, Stress-induced superoxide formation in *Bacillus cereus* detected using the fluorescent probe MitoSOX, *submitted for publication*.

Curriculum Vitae

Josephus Martinus Mols was born on August 24 1978 in Baarn. He finished his secondary education, receiving his HAVO diploma in 1995 and his VWO diploma in 1996 at the "Veluws College" in Apeldoorn. He studied Biology at Utrecht University specializing in Molecular Microbiology. As part of his MSc study, he contributed to two research projects. The first project was conducted in the Microbiology group of the Biology department of Utrecht University and involved a study of the outer membrane protein Omp85 in *Neisseria meningitides*, under the supervision of dr. R. Voulhoux and prof. dr. J. Tommassen. The second project, conducted at RIKILT Institute of Food Safety, dealt with comparative transcriptome analysis of *Salmonella* Typhimurium DT104 wild type and its *luxS* mutant under the supervision of dr. A. Hermans and prof. dr. T. Abee. Before receiving his MSc in Biology in August 2004, Maarten started his PhD project on the acid stress response of *Bacillus cereus*, at the Laboratory of Food Microbiology of Wageningen University in June 2004 under the supervision of prof. dr. T. Abee, prof. dr. ir. M.H. Zwietering, and dr. R. Moezelaar. This project was part of the TI Food & Nutrition (formerly known as Wageningen Centre for Food Sciences) project "Food preservation and safety" and focused on the identification of key factors in the acid stress response of *B. cereus* and aimed to gain a mechanistic understanding of the stress response. The approach used involved a combination of genomics, transcriptomics, and physiological responses, including flow cytometry analysis of radical formation using fluorescent probes. The results of this project are described in this thesis. As of August 2008, Maarten is employed as a post-doctoral researcher at the Laboratory of Food Microbiology of Wageningen University working on the Senter Novem EOS project "Mild sterilization using electric fields".

Overview of completed training activities

Discipline specific activities
Courses
VLAG Genetics and physiology of food-associated microorganisms (2004)
VLAG Bioinformatics course "Bioinformation Technology I" (2004)
Workshop 2D-electroforesis (2004)

Meetings
GRC General Stress Response, South Hadley, USA, (2006)
NVvM spring meeting, Papendal, (2007)
Bacillus-ACT 2007, Oslo, Norway (2007)
WCFS/ TIFN C009 Project meetings (2004, 2005, 2006, 2007, 2008)

General courses
VLAG PhD week, Bilthoven (2004)
OWU Afstudeervak organiseren en begeleiden (2006)
Presentation course "Leren presenteren", Wageningen (2006)
Course on making websites with CMS (2005)

Other activities
Preparation of PhD research proposal (2004)
WCFS Colloquia (2004, 2005)
WCFS/ TIFN Programme 3 WE-days (2004, 2005, 2006, 2007, 2008)
PhD/ Postdoc meetings, Laboratory of Food Microbiology (2004, 2005, 2007, 2008)
General meetings Laboratory of Food Microbiology (2004, 2005, 2006, 2007, 2008)
VLAG PhD trip Laboratory of Food Microbiology, South Africa (2005)
VLAG PhD trip Laboratory of Food Microbiology, Canada (2008)

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